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TITLE: A Counterregulatory Mechanism Impacting Androgen Suppression Therapy

PRINCIPAL INVESTIGATOR: Dr. David Wilson

Contracting Organization: Washington University, The
Saint Louis, MO 63130-4862

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Dr. David Wilson

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Washington University, The
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U.S. Army Medical Research Acquisition Act
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14. ABSTRACT

Androgen deprivation therapy (ADT) with gonadotropin-releasing hormone (GnRH) analogues is a mainstay of prostate cancer treatment. This project explores a novel counterregulatory response that may limit the efficacy of ADT. A key player in this process is HSD17B3, an enzyme required for the conversion of androstenedione to testosterone in testicular Leydig cells (LCs). Normally gonadotropin stimulation of LCs is accompanied by upregulation of genes in the testosterone synthetic pathway. The effect of GnRH analogues on LC function was modeled by conditional deletion of *Gata4*, a transcription factor known to positively regulate multiple genes involved in steroidogenesis. *Gata4* deletion led to decreased expression of several genes in the testosterone biosynthetic pathway (*Cyp11a1*, *Hsd3b1*, and *Cyp17a1*). Unexpectedly, the final gene in the pathway, *Hsd17b3*, was upregulated in the deleted cells. This paradoxical increase in *Hsd17b3* expression was recapitulated when normal LCs were incubated with conditioned medium from GATA4-deficient LCs, implying that a hormone mediates the process. Preliminary results suggest that a loss of LC-derived estrogen in the conditioned media accounts for the effect. If this counterregulatory mechanism also operates in human LCs, it could contribute to inadequate androgen suppression in patients who undergo ADT with GnRH analogues.

15. SUBJECT TERMS
androgen; estrogen; hydroxysteroid dehydrogenase; gonadotropin; Leydig cell; prostate cancer; steroidogenesis; testosterone

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Androgen deprivation therapy (ADT) with gonadotropin-releasing hormone (GnRH) analogues is a mainstay of prostate cancer treatment. This project explores a novel counterregulatory response that may limit the efficacy of ADT. A key player in this process is HSD17B3, an enzyme required for the conversion of androstenedione to testosterone in testicular Leydig cells (LCs). Normally gonadotropin stimulation of LCs is accompanied by upregulation of genes in the testosterone synthetic pathway. The effect of GnRH analogues on LC function was modeled by conditional deletion of Gata4, a transcription factor known to positively regulate multiple genes involved in steroidogenesis. Gata4 deletion led to decreased expression of several genes in the testosterone biosynthetic pathway (Cyp11a1, Hsd3b1, and Cyp17a1). Unexpectedly, the final gene in the pathway, Hsd17b3, was upregulated in the deleted cells. This paradoxical increase in Hsd17b3 expression was recapitulated when normal LCs were incubated with conditioned medium from GATA4-deficient LCs, implying that a hormone mediates the process. Preliminary results suggest that a loss of LC-derived estrogen in the conditioned media accounts for the effect. If this counterregulatory mechanism also operates in human LCs, it could contribute to inadequate androgen suppression in patients who undergo ADT with GnRH analogues.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Androgen; estrogen; hydroxysteroid dehydrogenase; gonadotropin; Leydig cell; prostate cancer; steroidogenesis; testosterone

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the USAMRAA Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Activity	Completion date or % completed
Specific Aim 1: Assess the impact of *Gata4* silencing on the expression of *Hsd17b3* in BLTK1 murine Leydig tumor cells and in primary cultures of murine adult LCs.	
Major Task 1: Perform a comprehensive biochemical analysis	
Subtask 1: Measurement of steroidogenic transcripts using qRT-PCR Cell lines used: primary murine adult LCs, BLTK1 cells	Sept 2015
Subtask 2: Measurement of enzyme activities Cell lines used: primary murine adult LCs, BLTK1 cells	50%
Subtask 3: Measurement of steroid hormone levels using LC-MS/MS Cell lines used: primary murine adult LCs, BLTK1 cells	50%
Milestone(s) Achieved: Measurement of the aforementioned items	70%
Specific Aim 2: Characterize the factor in conditioned medium that is responsible for the paradoxical upregulation of *Hsd17b3* expression in response to *Gata4* silencing.	
Major Task 2: Identify the paracrine or autocrine factor	
Subtask 1: Assess the impact of conditioned media on expression of *Hsd17b3* and other steroidogenic genes using different combinations of donor and recipient cells. Cell lines used: primary murine adult LCs, BLTK1 cells	Jan 2016
Subtask 2: Subject conditioned medium to simple biochemical purification strategies (dialysis, extraction, ammonium sulfate precipitation, etc). Cell lines used: primary murine adult LCs, BLTK1 cells	Feb 2016
Milestone(s) Achieved: Characterization of the factor in conditioned medium	Feb 2016

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Major activities: The focus of our work has been laboratory research. We have made significant progress on both Specific Aims of this 1-year exploratory grant, as detailed below.

2) Specific objectives:

Specific Aim 1: Assess the impact of *Gata4* silencing on the expression of *Hsd17b3* in BLTK1 murine Leydig tumor cells and in primary cultures of murine adult LCs.

Specific Aim 2: Characterize the factor in conditioned medium that is responsible for the paradoxical upregulation of *Hsd17b3* expression in response to *Gata4* silencing.

3) Significant results or key outcomes:

A. Gene silencing of *Gata4* in primary LCs leads to a paradoxical upreguation of *Hsd17b3* (Major Task 1, Subtask 1). Primary LCs were isolated from adult *Gata4*$^{flox/flox}$ mice. *In vitro* recombination of the floxed *Gata4* alleles was achieved by incubation with adenovirus expressing Cre-GFP; virus expressing GFP alone was used as a negative control. Following infection, cells were washed extensively and then incubated with fresh medium. GFP expression was used to assess infection efficiency. The levels of key steroidogenic transcripts were monitored by qRT-PCR. Early genes in the testosterone biosynthetic pathway (*Cyp11a1*, *Hsd3b1*, and *Cyp17a1*) were downregulated in response to *Gata4* deletion (Schrade et al., *Endocrinology*, 2015). In contrast, *Hsd17b3* was significantly upregulated (Figure 1).

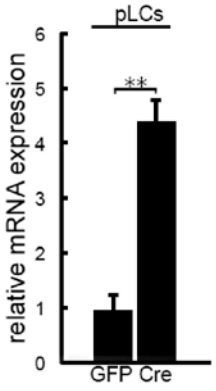

Figure 1. Paradoxical upreglation of *Hsd17b3* expression in response to *Gata4* gene silencing. Primary LCs (pLCs) from *Gata4*$^{flox/flox}$ mice were incubated with adenovirus expressing either GFP alone or Cre-GFP. Expression levels were measured by qRT-PCR 2 days later (**, $P < 0.01$).

Confirmatory analyses using enzyme assays (Major Task 1, Subtask 2) and steroid hormone measurements (Major Task 1, Subtask 3) are underway**.** These stated goals will be completed soon. A 3-month no-cost extention has been secured to support these efforts.

B. Gene silencing of *Gata4* in BLTK1 cells leads to a paradoxical upregulation of *Hsd17b3* (Major Task 1, Subtask 1). To independently validate the primary LC experiments, siRNA was used to silence *Gata4* in the BLTK1 murine Leydig tumor cell line. BLTK1 cells retain key LC characteristics including expression of *Hsd17b3* and production of testosterone. As in primary LCs, depletion of GATA4 in BLTK1 cells was associated with decreased expression of genes in the proximal testosterone biosynthetic pathway (*Cyp11a1*, *Hsd3b1*, and *Cyp17a1*) and increased expression of *Hsd17b3*. Because the basal and induced levels of *Hsd17b3* expression were much lower in BLTK1 cells than in primarly LCs, we opted to focus on primary cells for the ensuing experiments. Thus, the stated goals of performing enzyme assays (Major Task 1, Subtask 2) and steroid hormone measurements (Major Task 1, Subtask 3) on BLTK1 cells are were not pursued.

C. A soluble factor mediates this counterregulatory response in primary LCs (Major Task 2, Subtask 1)**.** The paradoxical increase in *Hsd17b3* expression was recapitulated when wild-type (WT) LCs (lacking floxed alleles) were incubated with conditioned medium from conditional knockout (cKO) LCs, suggesting that a secreted factor mediates the upregulation of *Hsd17b3* (Figure 2).

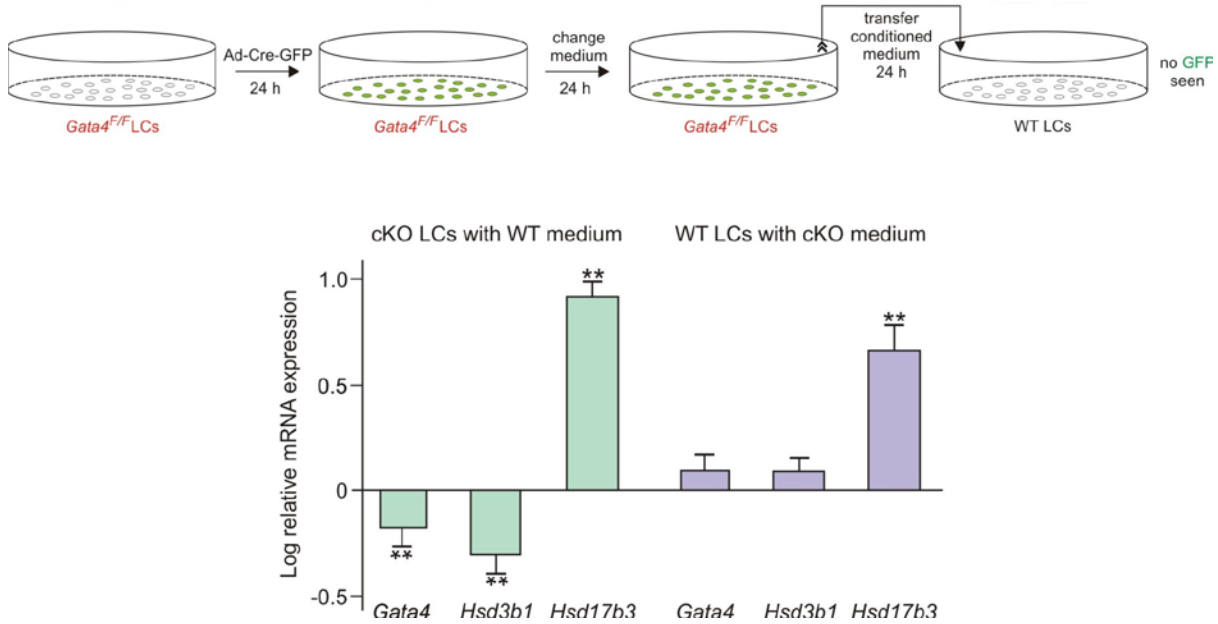

Figure 2. Reciprocal "supernatant swap" experiments show that a soluble factor mediates the upreglation of *Hsd17b3***.** Adult LCs were isolated from *Gata4*$^{flox/flox}$ (*Gata4*$^{F/F}$) and WT mice. *In vitro* recombination of floxed alleles was achieved by incubation with adenovirus expressing Cre-GFP or GFP alone. Following infection, cells were washed extensively and then incubated with fresh medium. Conditioned medium was harvested 24 h later from cKO cells later transferred to a separate dish of "reporter" WT cells. GFP expression was used to monitor infection efficiency and to control for cross-contamination of conditioned medium with adenovirus. The reciprocal experiment was conducted with conditioned media from WT cells and cKO reporter cells. Expression levels of key steroidogenic genes in the reporter cells were measured by qRT-PCR after 2 days of exposure to conditioned media (**, $P < 0.01$).

D. Preliminary results suggest that estrogen is the secreted factor that mediates this response (Major Task 2, Subtask 2). Mice harboring a null mutation in the estrogen receptor-α gene exhibit enhanced expression of *Hsd17b3*. Therefore, we hypothesized that the paradoxical upregulation of *Hsd17b3* expression could reflect a lack of estrogen production by the knockdown LCs. To test this possibility we repeated the supernatant swap experiment; the addition of 100 µM estradiol to conditioned medium from cKO cells mitigated the upregulation of *Hsd17b3* in WT reporter cells.

4) Other achievements: none

5) Future directions: In upcoming experiments that were not proposed in the original application, we will determine whether treatment of WT primary LCs with an aromatase inhibitor recapitulates the paradoxical upregulation of *Hsd17b3* seen with silencing of *Gata4*. To determine whether this upregulation occurs *in vivo*, we plan to inject the interstitial compartment of the testis in an anesthetized mouse with adenovirus expressing Cre-GFP; adenovirus expressing GFP alone will be injected as a negative control. Testicular mRNA will be harvested 2 days later and subjected to qRT-PCR analysis.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Nothing to Report

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Testosterone, a steroid hormone, plays a key role in the development, growth, and progression of prostate cancer. Testosterone is synthesized mainly by Leydig cells in the testis. The use of drugs to suppress testosterone production, a treatment termed chemical castration, is a mainstay of therapy for advanced prostate cancer. Unfortunately, these drugs do not adequately suppress testosterone production in a subset of men with prostate cancer, and this compromises their survival. Using mouse LCs, which are easier to isolated and manipulate than their human counterparts, we have shown that one of the key genes involved in testosterone synthesis, Hsd17b3, is subject to a distinctive form of counterregulation. If this same mechanism is operational in human LCs, it could limit the efficacy of ADT. This possibility justifies the further investigation of this phenomenon using both mouse models and cultured human LCs. The notion that decreased estrogen production by LCs triggers the counterregulatory response is provocative, particularly in light of the use of estrogen as an alternative form of ADT. Estrogen is presumed to act via feedback inhibition of the hypothalamic-pituitary-gonadal axis, but our results suggest that estrogen may have a direct inhibitory effect on expression testosterone biosynthesis. Thus, combination ADT with a GnRH analogue and estrogen might prove more effective than either drug alone.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Not Applicable

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

We secured a 3-month no-cost extension to complete the steroid hormone measurements. All the samples have been collected, but the mass spectrometric analyses have not been completed.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not Applicable

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Heikinheimo, M., Pihlajoki, M., Schrade, A., Kyrönlahti, A., Wilson, D. B. (2015). Testicular steroidogenic cells to the rescue. *Endocrinology*, 156 (5), 1616-1619; status of publication (published); acknowledgement of federal support (yes).

Pihlajoki, M., Färkkilä, A., Soini, T., Heikinheimo, M., Wilson, D. B. (2016). GATA factors in endocrine neoplasia. *Mol Cell Endocrinol*, 421, 2-17; status of publication (published); acknowledgement of federal support (yes).

Schrade, A., Kyrönlahti, A., Akinrinade, O., Pihlajoki, M., Hakkinen, M., Fischer, S., Alastalo, T. P., Velagapudi, V., Toppari, J., Wilson, D. B., Heikinheimo, M. (2015). GATA4 is a key regulator of steroidogenesis and glycolysis in mouse Leydig cells. *Endocrinology*, 156 (5), 1860-1872; status of publication (published); acknowledgement of federal support (yes).

Schrade, A., Kyrönlahti, A., Akinrinade, O., Pihlajoki, M., Fischer, S., Rodriguez, V. M., Otte, K., Velagapudi, V., Toppari, J., Wilson, D. B., Heikinheimo, M. (2016). GATA4 regulates blood-testis barrier function and lactate metabolism in mouse Sertoli cells. *Endocrinology*, en20151927; status of publication (accepted); acknowledgement of federal support (yes).

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Researchgate:

https://www.researchgate.net/profile/David_Wilson33

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Dr. David Wilson
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	ORCID ID 0000-0002-1826-7745
Nearest person month worked:	3
Contribution to Project:	Dr. Wilson oversaw experimental design and data interpretation.
Funding Support:	American Heart Association
Name:	Dr. Markku Heikinheimo
Project Role:	Unpaid consultant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	0
Contribution to Project:	Dr. Heikinheimo assisted with the gene silencing experiments
Funding Support:	Sigrid Jusélius Foundation Academy of Finland

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Completed grant:
American Heart Association, Grant-in-Aid, 13GRNT16850031
"Regulation of steroidogenic cell differentiation in the mouse"
David Wilson, PI
7/1/2013 - 6/30/2015

New award due to start later this year:
Department of Defense, Ovarian Cancer Research Program Pilot Award OC150105
"Ovarian Granulosa Cell Tumor: New Insights into the Clinical Challenge of Late Relapse"
David Wilson, PI
10/1/2016 - 9/30/2018

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other*

Organization name: University of Helsinki
Location: Helsinki, Finland
Contribution to the project: Collaboration (with Dr. Heikinheimo)

8. SPECIAL REPORTING REQUIREMENTS: NONE

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Curriculum Vitae

David Brian Wilson, M.D., Ph.D.

Date: March 15, 2016

Address and Telephone Numbers

University: Washington University School of Medicine
Department of Pediatrics
Division of Hematology and Oncology
660 South Euclid Avenue, Campus Box 8208
St. Louis, MO 63110
Phone: 314-286-2834
Fax: 314-454-2780
eMail: wilson_d@wustl.edu

Present Position

Professor of Pediatrics and Developmental Biology

Education and Training

1976 - 1980	B.A., Chemistry, Kalamazoo College, Kalamazoo, MI
1980 - 1986	M.D., Medicine, Washington University School of Medicine, St. Louis, MO
1980 - 1986	Ph.D., Biochemistry, Washington University School of Medicine, St. Louis, MO
1986 - 1988	Pediatric Resident, Boston Children's Hospital, Boston, MA
1988 - 1991	Hematology/Oncology Fellow, Dana Farber Cancer Inst, Boston, MA

Academic Positions and Employment

1991 - 1992 Instructor of Pediatrics, Harvard University, Boston, MA
1992 - 1997 Assistant Professor of Pediatrics and Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO
1997 - 2003 Director, Division of Pediatric Hematology/Oncology, Washington University School of Medicine, St. Louis, MO
1997 - 2015 Associate Professor of Pediatrics and Developmental Biology, Washington University School of Medicine, St. Louis, MO
2016 - Pres Professor of Pediatrics and Developmental Biology, Washington University, St. Louis, MO

Appointments and Committees

NIH Study Sections:

1995 - 2001 American Heart Association Great American Consortium (Standing Member)
1998 - 1999 NHLBI PPG (Non-Standing Member)
1998 - 1999 NIH Human Embryology and Development - 2 (Non-Standing Member)
2000 - 2010 Hope Street Kids Foundation (Standing Member)
2000 - 2001 NIH Special Emphasis Panel (CCVS 01) (Non-Standing Member)
2007 - 2008 NHLBI PPG (Non-Standing Member)
2009 - 2009 Medical Research Council, UK (Non-Standing Member)
2014 - Pres Alex's Lemonade Stand Foundation (Standing Member)
2014 - 2014 Department of Defense -- Bone Marrow Failure Research Program (Non-Standing Member)

Local Appointments:

1994 - Pres American Cancer Society, Intramural Grant Program, Study Section member
2013 - Pres Cancer Frontier Fund, Siteman Cancer Center, Study Section member

University Affiliations:

1996 - 2001 MSTP Admissions Committee
1997 - 2005 Head, American Cancer Society Intramural Grant Program
2006 - 2013 Benefits Committee
2008 - Pres Co-Leader, Developmental Biology and Genetics Research Unit
2010 - Pres Director, Student Exchange Program with Hochschule Mannheim --

University of Applied Sciences
2014 - Pres Co-Director, Pediatric Student Research Program (PSRP)

Hospital Affiliations:
1994 - 2002 Medical Director, St. Louis Children's Hospital Blood Bank
2014 - Pres BJC System Anticoagulation Committee

Scientific Advisory Boards:
2000 - 2010 Hope Street Kids (Pediatric Oncology Grants)

Thesis Committees (* Chair)		Advisor
1996 - 1997	Naoko Narita, M.D.	D. Wilson, M.D., Ph.D.
2001 - 2003	Alexander Ungewickle	P. Majerus, M.D.
2003 - 2005	David Grenda	D. Link, M.D.
2004 - 2006	Jesse Lugus	K.-C. Choi, Ph.D.
2006 - 2007	Ellen Langer	G. Longmore, M.D.
2006 - 2008	Erik Madsen	J. Gitlin, M.D.
2008 - 2009	Chin-Chen Liu	G. Bu, Ph.D.
2009 - 2010	Antti Kyrönlahti, M.D.	M. Heikinheimo, M.D., Ph.D
2009 - 2010	Justyna Krachulec*	D. Wilson, M.D., Ph.D.
2009 - 2011	Daniel Chen	P. Jay, M.D., Ph.D.
2010 - 2011	Anja Schrade*	D. Wilson, M.D., Ph.D.
2010 - 2013	Erica Schoeller	K. Moley, M.D.
2011 - 2012	Marjut Pihlajoki	M. Heikinheimo, M.D., Ph.D.
2011 - 2014	Maximilian Schillebeeckx	R. Mitra, Ph.D.
2012 - 2013	Elisabeth Gretzinger*	D. Wilson, M.D., Ph.D.
2012 -Pres	Anja Schrade	M. Heikinheimo, M.D., Ph.D.
2013 - 2014	Theresa Röhrig*	D. Wilson M.D., Ph.D.
2014 -Pres	Mark Valentine	T. Druley, M.D., Ph.D.
2014 - 2014	Claire Schulkey	P. Jay, M.D., Ph.D.
2014 - 2015	Julia Dörner*	D. Wilson, M.D., Ph.D.

Scholarship Oversight Committees
2008 - 2011 Ghada Kunter, M.D. (Advisor: D. Link, M.D.)
2008 - 2013 Todd Druley, M.D., Ph.D. (Advisor: R. Mitra, Ph.D.)
2009 - 2013 Laura Scheuttpelz M.D., Ph.D. (Advisor: D. Link, M.D.)
2010 - 2012 Edward Dela Ziga (Advisor: J. DiPersio M.D., Ph.D.)
2010 -Pres Todd Druley, M.D., Ph.D. (Advisor: R. Mitra, Ph.D.)
2010 - 2012 Mark Schroeder, M.D. (Advisor: J. DiPersio, M.D., Ph.D.)
2010 - 2012 Samuel Odame Anim, M.D. (Advisor: R. Strunk, M.D.)

2011 - 2012 Leili Dolatshahi, M.D. (Advisor: E. Sadler, M.D., Ph.D.)
2012 - 2014 Alexander Ngwube, M.D. (Advisor: M. Dinauer, M.D., Ph.D.)
2013 - 2015 Mark Levin, M.D. (Advisor: C. Nichols, Ph.D.)
2013 -Pres Monica Hulbert, M.D. (Advisor: R. Hayashi, M.D.)
2013 - 2015 Alok Kothari, M.D. (Advisor: G. Challen, Ph.D.)
2013 - 2015 Hoang Nguyen, M.D. (Advisor: P. Jay, M.D., Ph.D.)
2014 - 2015 Jennifer Heeley, M.D. (Advisor: B. Kozel, M.D., Ph.D.)

Licensure and Certifications

1993 - Pres MO Physician #103195
1993 - 2008 Pediatrics
1996 - 2015 Pediatric Hematology/Oncology
2015 - Pres IL Physician #036.139375

Honors and Awards

1980 Honors in Chemistry, Kalamazoo College
1980 Phi Beta Kappa
1981 Carter Prize for Achievement in the Medical Curriculum
1982 Lowry Prize in Pharmacology
1986 Alpha Omega Alpha
1992 McDonnell Scholars Award in Oncology
1992 Pfizer Junior Faculty Award in Cardiovascular Biology
1993 March of Dimes Basil O'Connor Award
1995 American Heart Association Established Investigator Award
1998 American Society for Clinical Investigation

Editorial Responsibilities

Editorial Boards:
2012 - 2016 Editorial Board, Mol Cell Endocrinol
2014 - 2016 Guest Editor, Special issue of Mol Cell Endocrinol, "Impact of maternal metabolism on newborn health"

Ad Hoc Reviewer:
1995 - Pres Development
1999 - Pres Dev Biol
2004 - Pres Pediatr Res

2007 - Pres Mol Cell Endocrinol
2008 - Pres Eur J Endocrinol
2008 - Pres Intl J Cancer
2008 - Pres J Mol Endocrinol
2008 - Pres Ped Blood Cancer
2011 - Pres Blood
2011 - Pres Histopathol
2011 - Pres Pediatr Pulmonol
2012 - Pres Andrology
2012 - Pres J Cell Sci
2012 - Pres PLoS One
2013 - Pres Endocrinology
2013 - Pres Histology and Histopathology
2013 - Pres Biol Repro
2013 - Pres Tumor Biology
2014 - Pres Hum Mol Genet
2014 - Pres Expert Review of Endocrinology & Metabolism
2014 - Pres Reproduction
2014 - Pres Cancer Causes & Control
2014 - Pres J Ped Hematol Oncol
2015 - Pres Frontiers in Endocrinology
2015 - Pres Am J Pathol
2015 - Pres Mol Biol Cell
2015 - Pres Nat Commun
2015 - Pres Stem Cells International
2015 - Pres Veterinary Quarterly

Professional Societies and Organizations

1994 - 2008 American Society for Biochemistry and Molecular Biology
1995 - Pres American Society for Hematology
1995 - Pres Pediatric Oncology Group/Children's Oncology Group
1998 - Pres American Heart Association
1998 - Pres American Society for Clinical Investigation
2012 - Pres Endocrine Society

Major Invited Professorships and Lectures

1993 Keystone Symposium in Cardiovascular Biology, Taos, NM

1995	AHA Symposium on Cardiac Development, New Orleans, LA
1997	Dana-Farber Cancer Institute 50th Anniversary Symposium, Boston, MA
2003	Finnish Endocrine Society, Espoo, Finland
2003	International GATA Factor Meeting, Montreal, Canada
2011	3rd International Conference on Adrenocortical Tumors, Würzburg, Germany
2014	Adrenal 2014 Symposium, Chicago, IL
2015	J. Lester Gabrilove Memorial Lecture, Mt. Sinai Hospital, New York, NY

Research Support

Governmental Support

Ovarian Cancer Research Program Pilot 10/1/2016 - 9/30/2018
Award OC150105
Department of Defense
Ovarian Granulosa Cell Tumor: New Insights into the Clinical Challenge of Late Relapse
This project uses a mouse model and patient data to explore the role of adjuvant therapy in the management of this tumor.
Role: Principal Investigator

T32 HD07499 (Alan Schwartz) 4/1/1999 - 3/31/2019
NIH
Training Program in Developmental Hematology
This training grant supports the education of physician-investigators in Hematology.
Role: Co-Principal Investigator

Completed Support

Scholars Program (David Wilson) 7/1/1992 - 6/30/1994
Pfizer, Inc.
Regulation of Preproendothelin-1 Gene Expression

Scholar's Award (David Wilson) 7/1/1992 - 6/1/1995
McDonnell Foundation
Regulation of Endothelial Cell Gene Expression

Basil O'Connor Award (David Wilson) 9/1/1993 - 8/31/1995
March of Dimes
Embryonic Heart Development

Basic Grant (David Wilson) 1/1/1994 - 12/31/1996

Monsanto-WU Biomedical Agreement
Biochemical Mechanisms of Cardiac Morphogenesis and Tissue Injury

Grant in Aid (David Wilson) 7/1/1994 - 6/30/1997
American Heart Association
Transcription Factors in Embryonic Heart Development

Basic Research Grant (David Wilson) 7/1/1996 - 6/30/1998
March of Dimes
Mammalian Yolk Sac Development

R01 HL52134 (David Wilson) 5/1/1994 - 4/30/1999
NIH
Embryonic Heart Development: Role of GATA-4

Basic Grant (David Wilson) 1/1/1997 - 12/31/1999
Monsanto-WU Biomedical Agreement
Regulation of Vascular Smooth Muscle by Transcription Factor GATA-6

Established Investigator Award (David Wilson) 7/1/1995 - 6/30/2000
American Heart Association
Cardiac Transcription Factor GATA-4 and the Disorganization Mouse

Grant in Aid (David Wilson) 1/1/1998 - 12/31/2000
American Heart Association
Endoderm-Mesoderm Interactions During Mouse Heart Development

P50 HL61001 (David Wilson) 1/1/1999 - 12/31/2003
NIH
Regulation and Expression of GATA Transcription Factors

Pilot Grant (David Wilson) 1/1/2003 - 12/1/2004
BJH Foundation
Molecular Basis of Familial Myelodysplastic Syndrome

Basic Research Grant (David Wilson) 6/1/2002 - 5/31/2005
March of Dimes
Role of GATA-4 in Stomach Development

Institutional Research Grant IRG-58-010-43 (David 7/1/1999 - 6/30/2005

Wilson)
American Cancer Society
Institutional Research Grant

Basic Research Grant (David Wilson) 4/1/2003 - 3/1/2006
Mallinckrodt Foundation
Molecular Basis of Bone Marrow Failure

Grant in Aid 0455623Z (David Wilson) 7/1/2004 - 6/30/2006
American Heart Association
Adrenocortical Cell Differentiation and Tumorigenesis

Pilot Translational Grant (Monica Bessler) 6/1/2009 - 5/31/2010
ICTS
Running the Stop in Bone Marrow Failure

Basic Research Grant MD-II-2009-174 (Monita Wilson) 2/1/2009 - 1/30/2012
Children's Discovery Institute
A Novel Mouse Model Linking Aberrant Inositol Cycling to Neural Tube Defects

R01 DK075618 (David Wilson) 4/1/2007 - 3/30/2013
NIH
Regulation of Steroidogenic Cell Differentiation in the Mouse

R01 CA105312 (Monica Bessler) 5/1/2004 - 4/30/2014
NIH
Molecular Determinants of Bone Marrow Failure

GIA 13GRNT16850031 (David Wilson) 7/1/2013 - 6/30/2015
American Heart Association
Regulation of steroidogenic cell differentiation in the mouse

Prostate Cancer Research Program 3/1/2015 - 2/29/2016
Exploratory-Hypothesis Development
Award PC141008 (David Wilson)
Department of Defense
A Counterregulatory Mechanism Impacting Androgen Suppression Therapy

Current Trainees

8/1/2011 Anja Schrade, M.Sc., Grad Student, Role of GATA factors in testicular development and function

8/30/2015 Ronni Manuel Götz, Rotation Student, Testicular steroidogenesis

Past Trainees

1994 - 2006 Markku Heikinheimo, M.D., Ph.D. (Other)
Study area: Research sabbatical; GATA4 in cardiac development
Present position: Professor, University of Helsinki, Helsinki, Finland

2008 - 2008 Simone Wagner, B.Sc. (Rotation Student)
Study area: Cyt b5 in adrenocortical tumors of the ferret
Present position: Staff scientist, Absolvent, Mannheim, Germany

2009 - 2009 Melanie Vetter, B.Sc. (Rotation Student)
Study area: GATA factors and steroidogenesis
Present position: PhD Candidate, Max Planck Institute of Biochemistry, Munich, Germany

2010 - 2010 Justyna Krachulec, M.Sc. (Grad Student)
Study area: Role of GATA4 in post-gonadectomy adrenocortical neoplasia
Present position: Graduate Student, Heidelberg University, Heidelberg, Germany

2011 - 2014 Maximilian Schillebeeckx, Ph.D. (Grad Student)
Study area: Epigenetics of adrenocortical neoplasia in the inbred mouse
Present position: Post-doctoral fellow, Washington University

2012 - 2012 Elisabeth Gretzinger, M.Sc. (Grad Student)
Study area: Mouse adrenocortical development
Present position: Staff Scientist, Pharmaceutical Company, Munich, Germany

2013 - 2013 Franziska Thol, B.Sc. (Rotation Student)
Study area: Genes implicated in adrenocortical development in the mouse
Present position: Student, Hochschule Mannheim -- University of Applied Sciences, Mannheim, Germany

2014 - 2014 Ricarda Ziegler, B.Sc. (Rotation Student)
Study area: Adrenocortical stem cell lineage tracing
Present position: Student, Hochschule Mannheim, Mannheim, Germany

2015 - 2015 Verena Martinez Rodriguez, B.Sc. (Rotation Student)
Study area: Hedgehog signaling in steroidogenic cells
Present position: Student, Hochschule Mannheim, Mannheim, Germany

1994 - 1996 Naoko Narita, M.D. (Postdoc Fellow)
Study area: Role of GATA factors in endoderm-mesoderm interactions

Present position: Associate Professor, Tsukuba University, Tsukuba, Japan

1997 - 2003 Christina Jacobsen, M.D., Ph.D. (Grad Student)
Study area: GATA factors in endoderm development and function
Present position: Instructor of Endocrinology, Boston Children's Hospital, Boston, MA

2009 - 2010 Antti Kyrönlahti, M.D., Ph.D. (Postdoc Fellow)
Study area: Role of GATA4 in ovarian and testicular development
Present position: Resident in Pediatrics, University of Helsinki Affiliated Hospitals, Helsinki, Finland

1994 - 1995 Patricia M. Gearhart, D.V.M., Ph.D. (Postdoc Fellow)
Study area: GATA transcription factor biology
Present position: Veterinary Ophthalmologist, Oakland Veterinary Referral Services , Bloomfield Hills, MI

2000 - 2003 Elena Genova, Ph.D. (Postdoc Fellow)
Study area: Regulation of transcription factor expression
Present position: Staff Scientist, Abbott Pharmaceuticals, Chicago, IL

2003 - 2004 Leslie A. Andritsos, M.D. (Postdoc Fellow)
Study area: TERC mutations in bone marrow failure
Present position: Assistant Professor of Internal Medicine, Ohio State University, Columbus, OH

2003 - 2005 Richard Peterson 2nd, D.V.M. (Postdoc Fellow)
Study area: Ferret adrenocortical tumors
Present position: Director, Molecular and Ultrastructural Pathology, GlaxoSmithKline, Raleigh-Durham, NC

2004 - 2006 Joshua Fields, M.D., M.S. (Clinical Research Trainee)
Study area: Molecular basis of bone marrow failure and pulmonary hypertension
Present position: Associate Professor, Department of Internal Medicine, Medical College of Wisconsin, Milwaukee, WI

2011 - 2012 Marjut Pihlajoki, M.Sc. (Grad Student)
Study area: Role of GATA factors in adrenocortical development and tumorigenesis
Present position: Graduate Student, University of Helsinki, Helsinki, Finland

2010 - 2011 Rosemarie Euler, B.Sc. (Rotation Student)
Study area: Role of GATA4 in steroidogenic cell development and function
Present position: Staff scientist, Pharmaceutical Industry, Mannheim, Germany

2012 - 2013 Theresa Hiller, B.Sc. (Rotation Student)
Study area: Novel markers of adrenocortical tumorigenesis in the mouse
Present position: Student, Hochschule Mannheim, Mannheim, Germany

2013 - 2014 Theresa Röhrig, M.Sc. (Grad Student)
Study area: Cell fate during adrenal development
Present position: Student, Mannheim University of Applied Sciences

2014 - 2015 Julia Dörner, M.Sc. (Graduate Student)
Study area: Adrenocortical stem cells
Present position: Student, Hochschule Mannheim, Mannheim, Germany

Clinical Responsibilities

1993 - Pres Attending physician, Hematology-Oncology, Pediatrics, Washington University School of Medicine and St. Louis Children's Hospital

2010 - Pres Director, Comprehensive Hemophilia Treatment Center, Pediatrics, Washington University and St. Louis Children's Hospital

2012 - Pres Attending Physician, Sickle Cell Program, Pediatrics, Washington University

Teaching Responsibilities

1995 - Pres Lecturer, "Chemotherapy" (1.5 hr/yr), Hematology-Oncology Pathobiology Course for 2nd year med students

1995 - Pres Teaching Laboratory (3 hr/yr), Hematology-Oncology Pathobiology Course for 2nd year med students

2003 - Pres Course Master, Special Emphasis Pathway in Cancer Biology L41 5196 BIOL (alternate years)

2011 - Pres Discussion Leader -- Hematology-Oncology Case Studies (8 hr/yr), Pediatric Clinical Clerkship Students and Residents

2014 - Pres Lecturer, "Transcription and mRNA processing", (1.5 hr/yr), Molecular Foundations of Medicine for 1st year med students

Publications

1. Jackson, R. L., Wilson, D.B., Glueck, C. J. (1979). Exchange of phospholipids between unilamellar vesicles of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine and plasma very low density lipoproteins. *Biochim Biophys Acta*, 557 (1), 79-85 PubMed: 232989.
2. Wilson, D. B., Ellsworth, J. L., Jackson, R. L. (1980). Net transfer of

phosphatidylcholine from plasma low density lipoproteins to sphingomyelin-apolipoprotein A-II complexes by bovine liver and human plasma phospholipid exchange proteins. *Biochim Biophys Acta*, 620 (3), 550-61 PubMed: 7236658.

3. Wilson, D. B., Prescott, S. M., Majerus, P. W. (1982). Discovery of an arachidonoyl coenzyme A synthetase in human platelets. *J Biol Chem*, 257 (7), 3510-5 PubMed: 7061494.
4. Neufeld, E. J., Wilson, D. B., Sprecher, H., Majerus, P. W. (1983). High affinity esterification of eicosanoid precursor fatty acids by platelets. *J Clin Invest*, 72 (1), 214-20. PMCID: PMC1129176 PubMed: 6308046.
5. Wilson, D. B., Salem, H. H., Mruk, J. S., Maruyama, I., Majerus, P. W. (1984). Biosynthesis of coagulation Factor V by a human hepatocellular carcinoma cell line. *J Clin Invest*, 73 (3), 654-8. PMCID: PMC425065 PubMed: 6200498.
6. Wilson, D. B., Bross, T. E., Hofmann, S. L., Majerus, P. W. (1984). Hydrolysis of polyphosphoinositides by purified sheep seminal vesicle phospholipase C enzymes. *J Biol Chem*, 259 (19), 11718-24 PubMed: 6090445.
7. Wilson, D. B., Neufeld, E. J., Majerus, P. W. (1985). Phosphoinositide interconversion in thrombin-stimulated human platelets. *J Biol Chem*, 260 (2), 1046-51 PubMed: 2981829.
8. Wilson, D. B., Bross, T. E., Sherman, W. R., Berger, R. A., Majerus, P. W. (1985). Inositol cyclic phosphates are produced by cleavage of phosphatidylphosphoinositols (polyphosphoinositides) with purified sheep seminal vesicle phospholipase C enzymes. *Proc Natl Acad Sci U S A*, 82 (12), 4013-7. PMCID: PMC397924 PubMed: 2987959.
9. Auchus, R. J., Wilson, D. B., Covey, D. F., Majerus, P. W. (1985). The 5-hydroxyl of myo-inositol is essential for uptake into HSDM1C1 mouse fibrosarcoma cells. *Biochem Biophys Res Commun*, 130 (3), 1139-46 PubMed: 4026861.
10. Wilson, D. B., Connolly, T. M., Bross, T. E., Majerus, P. W., Sherman, W. R., Tyler, A. N., Rubin, L. J., Brown, J. E. (1985). Isolation and characterization of the inositol cyclic phosphate products of polyphosphoinositide cleavage by phospholipase C. Physiological effects in permeabilized platelets and Limulus photoreceptor cells. *J Biol Chem*, 260 (25), 13496-501 PubMed: 2997167.
11. Connolly, T. M., Wilson, D. B., Bross, T. E., Majerus, P. W. (1986). Isolation and characterization of the inositol cyclic phosphate products of phosphoinositide cleavage by phospholipase C. Metabolism in cell-free extracts. *J Biol Chem*, 261 (1), 122-6 PubMed: 3001044.
12. Wilson, D. B., Dorfman, D. M., Orkin, S. H. (1990). A nonerythroid GATA-binding protein is required for function of the human preproendothelin-1 promoter in endothelial cells. *Mol Cell Biol*, 10 (9), 4854-62. PMCID: PMC361096 PubMed: 2388628.

13. Dorfman, D. M., Wilson, D. B., Bruns, G. A., Orkin, S. H. (1992). Human transcription factor GATA-2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. *J Biol Chem*, 267 (2), 1279-85 PubMed: 1370462.
14. Wilson, D. B., Wilson, M. P. (1992). Identification and subcellular localization of human rab5b, a new member of the ras-related superfamily of GTPases. *J Clin Invest*, 89 (3), 996-1005. PMCID: PMC442949 PubMed: 1541686.
15. Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H., Wilson, D. B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol*, 13 (4), 2235-46. PMCID: PMC359544 PubMed: 8455608.
16. Heikinheimo, M., Scandrett, J. M., Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev Biol*, 164 (2), 361-73 PubMed: 8045339.
17. Heikinheimo, M., Lawshé, A., Shackleford, G. M., Wilson, D. B., MacArthur, C. A. (1994). Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. *Mech Dev*, 48 (2), 129-38 PubMed: 7873403.
18. Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C. N., Simon, M. C., Leiden, J. M., Parmacek, M. S. (1994). The GATA-4 transcription factor transactivates the cardiac muscle-specific troponin C promoter-enhancer in nonmuscle cells. *Mol Cell Biol*, 14 (11), 7517-26. PMCID: PMC359288 PubMed: 7935467.
19. Ball, R. H., Picus, D., Goyal, R. K., Wilson, D. B., Rader, J. S. (1995). Ovarian artery pseudoaneurysm: diagnosis by Doppler sonography and treatment with transcatheter embolization. *J Ultrasound Med*, 14 (3), 250-2 PubMed: 7760472.
20. Bielinska, M., Wilson, D. B. (1995). Regulation of J6 gene expression by transcription factor GATA-4. *Biochem J*, 307 (Pt 1), 183-9. PMCID: PMC1136761 PubMed: 7717974.
21. White, R. A., Dowler, L. L., Pasztor, L. M., Gatson, L. L., Adkison, L. R., Angeloni, S. V., Wilson, D. B. (1995). Assignment of the transcription factor GATA4 gene to human chromosome 8 and mouse chromosome 14: Gata4 is a candidate gene for Ds (disorganization). *Genomics*, 27 (1), 20-6 PubMed: 7665171.
22. Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M., Wilson, D. B. (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development*, 121 (11), 3877-88 PubMed: 8582296.
23. Narita, N., Heikinheimo, M., Bielinska, M., White, R. A., Wilson, D. B. (1996). The gene for transcription factor GATA-6 resides on mouse chromosome 18 and is expressed in myocardium and vascular smooth muscle. *Genomics*, 36 (2), 345-8

PubMed: 8812463.
24. Goyal, R. K., McEvoy, L., Wilson, D. B. (1996). Hodgkin disease after renal transplantation in childhood. *J Pediatr Hematol Oncol*, 18 (4), 392-5 PubMed: 8888750.
25. Bielinska, M., Narita, N., Heikinheimo, M., Porter, S. B., Wilson, D. B. (1996). Erythropoiesis and vasculogenesis in embryoid bodies lacking visceral yolk sac endoderm. *Blood*, 88 (10), 3720-30 PubMed: 8916936.
26. Bielinska, M., Wilson, D. B. (1997). Induction of yolk sac endoderm in GATA-4-deficient embryoid bodies by retinoic acid. *Mech Dev*, 65 (1-2), 43-54 PubMed: 9256344.
27. Heikinheimo, M., Ermolaeva, M., Bielinska, M., Rahman, N. A., Narita, N., Huhtaniemi, I. T., Tapanainen, J. S., Wilson, D. B. (1997). Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. *Endocrinology*, 138 (8), 3505-14 PubMed: 9231805.
28. Narita, N., Bielinska, M., Wilson, D. B. (1997). Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev Biol*, 189 (2), 270-4 PubMed: 9299119.
29. Narita, N., Bielinska, M., Wilson, D. B. (1997). Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. *Development*, 124 (19), 3755-64 PubMed: 9367431.
30. Ketola, I., Rahman, N., Toppari, J., Bielinska, M., Porter-Tinge, S. B., Tapanainen, J. S., Huhtaniemi, I. T., Wilson, D. B., Heikinheimo, M. (1999). Expression and regulation of transcription factors GATA-4 and GATA-6 in developing mouse testis. *Endocrinology*, 140 (3), 1470-80 PubMed: 10067876.
31. Pehlivan, T., Pober, B. R., Brueckner, M., Garrett, S., Slaugh, R., Van Rheeden, R., Wilson, D. B., Watson, M. S., Hing, A. V. (1999). GATA4 haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. *Am J Med Genet*, 83 (3), 201-6 PubMed: 10096597.
32. Bielinska, M., Narita, N., Wilson, D. B. (1999). Distinct roles for visceral endoderm during embryonic mouse development. *Int J Dev Biol*, 43 (3), 183-205 PubMed: 10410899.
33. Kiiveri, S., Siltanen, S., Rahman, N., Bielinska, M., Lehto, V. P., Huhtaniemi, I. T., Muglia, L. J., Wilson, D. B., Heikinheimo, M. (1999). Reciprocal changes in the expression of transcription factors GATA-4 and GATA-6 accompany adrenocortical tumorigenesis in mice and humans. *Mol Med*, 5 (7), 490-501. PMCID: PMC2230442 PubMed: 10449810.
34. Siltanen, S., Anttonen, M., Heikkilä, P., Narita, N., Laitinen, M., Ritvos, O., Wilson, D. B., Heikinheimo, M. (1999). Transcription factor GATA-4 is expressed in pediatric yolk sac tumors. *Am J Pathol*, 155 (6), 1823-9. PMCID: PMC1866939 PubMed: 10595911.

35. Laitinen, M. P., Anttonen, M., Ketola, I., Wilson, D. B., Ritvos, O., Butzow, R., Heikinheimo, M. (2000). Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors. *J Clin Endocrinol Metab*, 85 (9), 3476-83 PubMed: 10999851.
36. Jacobsen, C. M., Narita, N., Bielinska, M., Syder, A. J., Gordon, J. I., Wilson, D. B. (2002). Genetic mosaic analysis reveals that GATA-4 is required for proper differentiation of mouse gastric epithelium. *Dev Biol*, 241 (1), 34-46 PubMed: 11784093.
37. Kiiveri, S., Liu, J., Westerholm-Ormio, M., Narita, N., Wilson, D. B., Voutilainen, R., Heikinheimo, M. (2002). Differential expression of GATA-4 and GATA-6 in fetal and adult mouse and human adrenal tissue. *Endocrinology*, 143 (8), 3136-43 PubMed: 12130579.
38. Kiiveri, S., Liu, J., Westerholm-Ormio, M., Narita, N., Wilson, D. B., Voutilainen, R., Heikinheimo, M. (2002). Transcription factors GATA-4 and GATA-6 during mouse and human adrenocortical development. *Endocr Res*, 28 (4), 647-50 PubMed: 12530677.
39. Clabby, M. L., Robison, T. A., Quigley, H. F., Wilson, D. B., Kelly, D. P. (2003). Retinoid X receptor alpha represses GATA-4-mediated transcription via a retinoid-dependent interaction with the cardiac-enriched repressor FOG-2. *J Biol Chem*, 278 (8), 5760-7 PubMed: 12480945.
40. Wilson, D. B., Ivanovich, J., Whelan, A., Goodfellow, P. J., Bessler, M. (2003). Human telomerase RNA mutations and bone marrow failure. *Lancet*, 361 (9373), 1993-4 PubMed: 12801777.
41. Bielinska, M., Parviainen, H., Porter-Tinge, S. B., Kiiveri, S., Genova, E., Rahman, N., Huhtaniemi, I. T., Muglia, L. J., Heikinheimo, M., Wilson, D. B. (2003). Mouse strain susceptibility to gonadectomy-induced adrenocortical tumor formation correlates with the expression of GATA-4 and luteinizing hormone receptor. *Endocrinology*, 144 (9), 4123-33 PubMed: 12933687.
42. Hostetler, M. A., Dribben, W., Wilson, D. B., Grossman, W. J. (2003). Sudden unexplained hemolysis occurring in an infant due to presumed Loxosceles envenomation. *J Emerg Med*, 25 (3), 277-82 PubMed: 14585455.
43. Wilson, D. B., Michalski, J. M., Grossman, W. J., Hayashi, R. J. (2003). Isolated CNS relapse following stem cell transplantation for juvenile myelomonocytic leukemia. *J Pediatr Hematol Oncol*, 25 (11), 910-3 PubMed: 14608204.
44. Grossman, W. J., Wilson, D. B. (2004). Hypopigmentation from imatinib mesylate (Gleevec). *J Pediatr Hematol Oncol*, 26 (3), 214 PubMed: 15125619.
45. Wilson, D. B., Rao, A., Hulbert, M., Mychaliska, K. P., Luchtman-Jones, L., Hill, D. A., Foglia, R. P. (2004). Neutropenic enterocolitis as a presenting complication of acute lymphoblastic leukemia: an unusual case marked by delayed perforation of the descending colon. *J Pediatr Surg*, 39 (7), e18-20 PubMed: 15213940.

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GATA4 regulates blood-testis barrier function and lactate metabolism in mouse Sertoli cells

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Conditional deletion of *Gata4* in Sertoli cells (SCs) of adult mice has been shown to increase permeability of the blood-testis barrier (BTB) and disrupt spermatogenesis. To gain insight into the molecular underpinnings of these phenotypic abnormalities, we assessed the impact of *Gata4* gene silencing in cell culture models. Microarray hybridization identified genes dysregulated by siRNA-mediated inhibition of *Gata4* in TM4 cells, an immortalized mouse SC line. Differentially expressed genes were validated by qRT-PCR analysis of primary cultures of *Gata4*$^{flox/flox}$ mouse SCs that had been subjected to cre-mediated recombination *in vitro*. Depletion of GATA4 in TM4 cells and primary SCs was associated with altered expression of genes involved in key facets of BTB maintenance, including tight/adherens junction formation (*Tjp1*, *Cldn12*, *Vcl*, *Tnc*, *Csk*) and extracellular matrix reorganization (*Lamc1*, *Col4a1*, *Col4a5*, *Mmp10*, *Mmp23*, *Timp2*). Western blotting and immunocytochemistry demonstrated reduced levels of TJP1, a prototypical tight junction protein, in GATA4-depleted cells. These changes were accompanied by a loss of morphologically-recognizable junctional complexes and a decline in trans-epithelial membrane resistance. Furthermore, *Gata4* gene silencing was associated with altered expression of *Hk1*, *Gpi1*, *Pfkp*, *Pgam1*, *Gls2*, *Pdk3*, *Pkd4*, and *Ldhb*, genes regulating the production of lactate, a key nutrient that SCs provide to developing germ cells. Comprehensive metabolomic profiling demonstrated impaired lactate production in GATA4-deficient SCs. We conclude that GATA4 plays a pivotal role in the regulation of BTB function and lactate metabolism in mouse SCs.

Sertoli cells (SCs) provide a microenvironment that facilitates spermatogenesis, the maturation of germ cells within the seminiferous tubules. A key component of this microenvironment is the blood-testis barrier (BTB), a dynamic structure composed of tight junctions, adherens junctions, gap junctions, and other protein complexes that link adjacent SCs (1, 2). The BTB partitions the seminiferous epithelium into two distinct milieus: 1) a basal compartment that is in contact with the systemic circulation and harbors spermatogonial stem cells (SSCs) plus spermatogonia, and 2) an apical compartment that is isolated from the systemic circulation and contains meiotic and postmeiotic germ cells (2, 3). The BTB undergoes remodeling to permit the passage of differentiating germ cells from the basal to apical compartment (1, 4). SC-derived extracellular matrix (ECM) proteins regulate junction dynamics during spermatogenesis (5). These proteins act in concert with proteases, protease inhibitors, focal adhesion proteins, and cytokines to regulate cell-cell interactions and maintain functional barrier integrity (5).

In addition to providing a structural framework for spermatogenesis, SCs afford trophic support for germ cell

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Abbreviations:

development. SCs secrete growth factors and chemokines that promote SSC self-renewal and maintenance (6–9). SCs also regulate the flow of essential nutrients to germ cells in the apical compartment (10). Whereas spermatogonia use glucose as a fuel for adenosine triphosphate (ATP) production, more developed germ cells, such as spermatocytes and spermatids, rely on SC-derived lactate as an energy source (11). To ensure adequate lactate production, SCs adopt a metabolic profile typical of cancer cells, the so-called Warburg phenotype, wherein most pyruvate generated through glycolysis is converted to lactate rather than being oxidized via the tricarboxylic acid (TCA) cycle (10, 12–15). SC lactate production is augmented further through catabolism of certain amino acids, notably glutamine (10). For their own energy needs, SCs rely on ATP derived from the β-oxidation of fatty acids (15, 16).

Studies of genetically-engineered mice have implicated GATA4, a transcription factor expressed in SCs and other testicular somatic cells, in the structural and trophic support of spermatogenesis (17–19). Conditional ablation of *Gata4* at E10.5 with *Wt1*-creER^{T2} impairs SC differentiation and causes male-to-female sex reversal, while deletion of *Gata4* at E12.5 using *Sf1*-cre leads to testis cord defects and decreased expression of another sex determination gene, *Dmrt1*, in SCs (20). Ablation of *Gata4* in fetal and neonatal SCs using *Amh*-cre disrupts the SSC niche and triggers germ cell depletion by impairing chemokine signaling (9). The gradual deletion of *Gata4* in the SCs of adult mice using *Amhr2*-cre leads to increased permeability of the BTB, selective loss of late stage (haploid) germ cells, and late-onset testicular atrophy with loss of fertility (19).

Although mutant mouse studies provide compelling evidence that GATA4 regulates SC development and function, the molecular pathways involved are not well understood, particularly in SCs of the adult. This is due in part to the inherent challenges of interpreting conditional knockout studies in the mouse testis. As reviewed elsewhere (17), cellular heterogeneity, compensatory responses, and other factors confound the analysis of such experiments. To circumvent these limitations, we have assessed the impact of GATA4 deficiency on SC function in less complicated experimental systems: a mouse SC line (TM4) and primary cultures of adult mouse SCs (pSCs). Using complementary methods, including transcriptomic and metabolomic analyses, we show that *Gata4* silencing disrupts specific aspects of SC function, notably BTB maintenance and lactate metabolism.

Materials and Methods

Animals and cultured cells

Experiments involving mice were approved by the Animal Studies Committee at Washington University. *Gata4*$^{flox/flox}$ mice (also termed *Gata4*$^{tm1.1Sad}$/J) (21, 22) were purchased from The Jackson Laboratory (Bar Harbor, ME). pSCs were isolated from 3- to 6-month-old *Gata4*$^{flox/flox}$ or wild-type (WT) 129.B6 mice using Percoll density separation (23) and maintained in DMEM/F12+GlutaMAX media supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, and 100 mg/L penicillin/streptomycin (all from Life Technologies, Grand Island, NY). Preparations of pSCs were determined to be 90%–95% pure on the basis of immunostaining for the SC marker RHOX5 and the Leydig cell marker 3βHSD (24). Mouse TM4 cells (25) were cultured in DMEM/F12+GlutaMAX media supplemented with 10% FBS, 25 mM HEPES, and 100 mg/L penicillin/streptomycin.

Knockdown of *Gata4* in TM4 cells and primary adult Sertoli cells

TM4 cells (passages 12 to 18) and WT pSCs were transiently transfected in the absence of antibiotics with a pool of 4 siRNAs targeting *Gata4* (5′-AGAGAAUAGCUUCGAACCA-3′, 5′-GGAUAUGGGUGUUCCGGGU-3′, 5′-CUGAAUAAAUCUAAGACGC-3′, 5′-GGACAUAAUCACCGCGUAA-3′) or with nontargeting control siRNA (5′-UGGUUUACAUGUCGACUAA-3′; all from Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX transfection reagent in Opti-MEM (Life Technologies) at a final concentration of 0.1 μM. Conditioned media and cells were collected 72 hours post-transfection for the analyses described below. pSCs from *Gata4*$^{flox/flox}$ mice were cultured in the presence of adenovirus [multiplicity of infection (MOI) = 100] expressing either green fluorescent protein (Ad-GFP) or the combination of cre recombinase and GFP (Ad-cre-IRES-GFP) (Vector Biolabs, Philadelphia, PA). Following infection, the cells were maintained in serum free DMEM/F12+GlutaMAX (Life Technologies) for 24 hours before RNA extraction.

Quantitative reverse transcriptase-PCR (qRT-PCR)

Total RNA was isolated using the Nucleospin RNA/Protein kit (Machrey-Nagel, Düren, Germany) and reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies). qRT-PCR was performed using SYBR GREEN I (Invitrogen, Carlsbad, CA), and expression was normalized to the housekeeping genes *Actb* and *L19*. Primer pairs are listed in Supplemental Table 1.

Western blotting

Protein was extracted from cell cultures with the NucleoSpin RNA/Protein kit (Machrey-Nagel), and 20 μg of protein was separated by SDS-PAGE and transferred onto a PVDF membrane (Invitrogen). A list of antibodies used is provided in Supplemental Table 2. The Immun-Star WesternC kit (Bio-Rad, Hercules, CA) was used for detection. Quantity One 1-D Analysis Software was used to determine quantitative protein signals.

Immunocytochemistry and immunofluorescence

TM4 cells and pSCs were grown on 4-well glass Lab Tek Chamber Slides (Sigma, St. Louis, MO) and fixed 72 hours post-transfection or 48 hours postinfection with 4% paraformaldehyde (PFA) in PBS. Immunoperoxidase and indirect immunofluorescent staining were performed as described (26). See Supplemental Table 2 for a list of antibodies.

Transmission electron microscopy (EM)

TM4 cells were grown on 4-well Permanox matrigel coated chamber slides (Sigma) and fixed 72 hours post-transfection with modified Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer) for 1 hour. Samples were postfixed in 2% OsO_4 in 0.1M sodium cacodylate buffer for 1 hour. The samples were then dehydrated and embedded in epon. Thick sections (1 μm) were stained with toluidine blue and examined by light microscopy to determine which blocks were to be thin-sectioned (90 nm). Thin sections were stained with uranyl acetate and lead citrate and examined by EM using a Model 1400EX electron microscope (EM) (JEOL, Tokyo, Japan).

Microarray expression profiling and gene set enrichment analysis

RNA was isolated from TM4 cells 72 hours after transfection with *Gata4* or nontargeting siRNA (n = 3) using NucleoSpin RNA/Protein kit and purified with NucleoSpin RNA Clean-up XS kit (Machrey-Nagel, Düren, Germany). RNA quality was assessed via Bioanalyzer (Agilent, Santa Clara, CA). Array hybridization was performed by the Functional Genomics Unit at the University of Helsinki using an Illumina MouseWG-6 v2.0 oligonucleotide BeadChip. Data was background corrected using BeadStudio software (Illumina, San Diego, CA); quantile normalization and log2 transformation were performed using the BeadArray bioconductor package (27). Differentially expressed genes were identified using LIMMA [linear models for microarray data (28)] with Benjamini-Hochberg correction. Expression levels with at least 1.5x difference and a false discovery rate (FDR) below 5% were considered as significantly differentially expressed. Microarray data was subjected to average linkage clustering with uncentered correlation using Cluster (29). Gene set enrichment analysis of the differentially expressed genes was performed using GOstats bioconductor package (30). Hypergeometric tests with the Benjamini-Hochberg FDR were performed to adjust the *P*-value.

Transepithelial resistance measurements

To assess barrier integrity, TM4 cells, *Gata4*$^{flox/flox}$ pSCs, and WT pSCs were treated either with siRNA or adenovirus, as described above, and then plated at a density of 0.5×10^6 cells/cm^2 (TM4) or 1.2×10^6 cells/cm^2 (*Gata4*$^{flox/flox}$ and WT pSCs) on matrigel-coated bicameral culture units (Merck Millipore, Billerica, MA) (31). Cells were incubated in a humidified CO_2 incubator at 37°C and transepithelial resistance (TER) was measured every 12 hours using the Millicell Electrical Resistance System with Ag/AgCl electrodes as described (31).

Cell viability assay

Cell viability was assayed using CellTiter 96 Aqueous One Solution (Promega, Madison, WI) at 24 hours, 48 hours, and 72 hours post transfection. Absolute absorbance (490 nm) was normalized using values obtained from wells containing nontransfected TM4 cells. To control for cell number, TM4 cells, *Gata4*$^{flox/flox}$ pSCs, and WT pSCs that had been subjected to *Gata4* gene silencing were trypsinized and counted every 24 hours for 6 days using a hemocytometer.

Metabolomic profiling

Metabolites were extracted from cell samples (n = 4), separated using Acquity UPLC, and analyzed using XEVO TQ-S Triple Quadrupole liquid chromatography/mass spectrometry (LC/MS; Waters Corporation, Milford, MA). At 72 hours post-transfection, ~2 million TM4 cells per sample were washed with PBS and deionized water, and subsequently quenched in liquid nitrogen. Metabolites were extracted by adding 20 μL of labeled internal standard mix and 1 ml of cold extraction solvent (80/20 acetonitrile/H_2O + 1% formic acid). Extracts were vacuum filtered (δ pressure 300–400 mbar for 2.5 minutes; Hamilton, Reno, NV) and injected into the LC system. A detailed description of instrument parameters is given elsewhere (42). A total of 110 metabolite concentrations were measured and data were normalized and analyzed using Metaboanalyst 3.0 software.

Quantification of glucose, lactate, and ammonium concentrations in conditioned media

Conditioned cell culture media were collected 72 hours post-transfection (TM4 cells) or 48 hours post infection (*Gata4*$^{flox/flox}$ pSCs), and glucose, lactate, and ammonium concentrations were measured with Konelab Arena 20 XT (Thermo Electron Oy, Helsinki, Finland; n = 4–7) as described (32).

Lactate dehydrogenase (LDH) activity measurements

LDH activity in TM4 cells, *Gata4*$^{flox/flox}$ pSCs, and WT pSCs was determined using the Promega CytoTox96 assay following the manufacturer's instructions. The assay was calibrated with the positive control included in the kit, and measurements (absorbance at 492 nm) were normalized to the number of cells, with values expressed as fold variation relative to the control group.

Statistical methods

mRNA levels, absorbance values for viability assays, cell counts, luminescence intensities, and metabolite concentrations in conditioned media were analyzed using the Student's *t* test or when appropriate, one-way ANOVA followed by Dunnett's test. Statistical significance was set at: * = $P < .05$, and ** = $P < .01$.

Results

GATA4-depleted SCs exhibit dysregulation of genes involved the formation and remodeling of junctional complexes

We used siRNA to inhibit *Gata4* expression in mouse TM4 cells, an immortalized cell line that retains many of

the properties of endogenous SCs and is easier to maintain and manipulate in culture than pSCs (33). To determine the efficiency of gene silencing, RNA and protein were isolated from TM4 cells 72 hours after siRNA transfection. *Gata4* mRNA levels were reduced by 78 ± 3% in cells treated with *Gata4* siRNA vs. nontargeting siRNA treated cells (n = 4; $P < .01$) (Figure 1A). Western blotting demonstrated only a trace of residual GATA4 protein in the *Gata4* siRNA treated cells (Figure 1B), and immunocytochemistry confirmed markedly reduced GATA4 staining in the nuclei of the targeted cells (Figure 1C-D).

Microarray hybridization was used to assess the impact of *Gata4* silencing on the TM4 transcriptome (complete results are available via GEO accession number GSE74471). A total of 2414 probes were differentially expressed (1,230 upregulated, 1184 downregulated). Results were ranked according to their log2 fold change (log FC) values. Unsupervised hierarchical clustering of the top 50 differentially expressed probes is shown in Figure 2. To identify biological processes affected by inhibition of *Gata4* in TM4 cells, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) analysis with all differentially expressed probes (Table 1). Among the terms identified in the KEGG pathway and GO analyses were processes linked to BTB function, such as focal adhesion, ECM receptor interaction, adherens junctions, and extracellular structure organization (1, 4, 5).

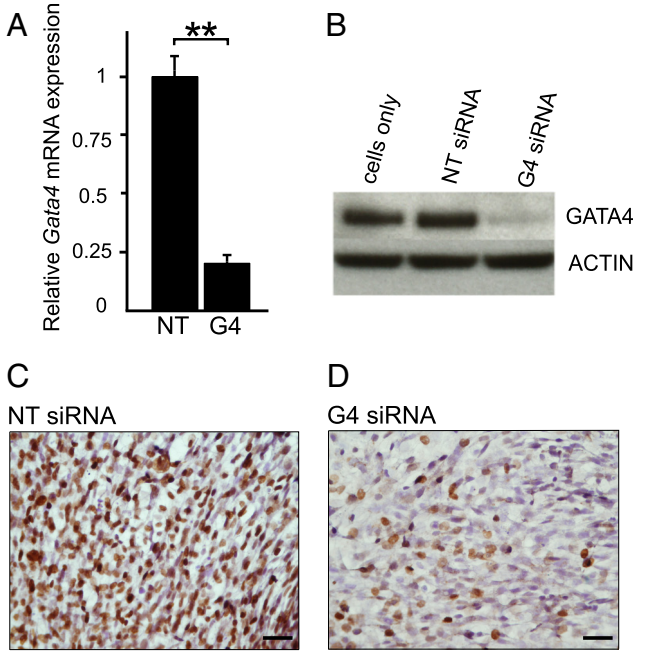

Figure 1. siRNA mediated inhibition of *Gata4* expression in TM4 cells. Cells were treated with nontargeting (NT) siRNA or *Gata4* (G4) siRNA for 72 hours (A-D). The efficiency of gene silencing was determined using qRT-PCR, western blotting, and immunocytochemistry. A, qRT-PCR results, normalized to *Actb* and *L19* mRNA, presented as mean relative expression values ± S.D. (**, $P < .01$, n = 4). B, Western blot analysis with Actin antibody as a control. (C, D) GATA4 immunoperoxidase staining of TM4 cells exposed to NT siRNA or G4 siRNA, respectively. Cells were counterstained with hematoxylin. Bars = 50 μm.

To confirm the microarray results, qRT-PCR analysis was performed on RNA isolated from TM4 cells transfected with *Gata4* siRNA or nontargeting siRNA. As further validation, we assessed the impact of *Gata4* inhibition on pSCs from adult mice. *Gata4* expression in pSCs was inhibited either via siRNA or through cre-mediated recombination. For the latter approach SCs isolated from *Gata4*$^{flox/flox}$ mice were infected in vitro with the cre-expressing adenoviral vector Ad-cre-IRES-GFP or the control vector Ad-GFP. Based on GFP expression, the infection efficiency of the adrenoviral vectors was determined to be > 90%. qRT-PCR analysis showed that infection of *Gata4*$^{flox/flox}$ pSCs with Ad-cre-IRES-GFP vs. Ad-GFP resulted in 58 ± 10% inhibition of *Gata4* at 48 hours post infection (n = 4; $P < .01$) (Supplemental Figure 1A). Treatment of WT pSCs with *Gata4* siRNA resulted in 81 ± 6% inhibition of *Gata4* (n = 4; $P < .01$) (Supplemental Figure 1A). The loss of GATA4 protein in cre-recombined or siRNA-treated pSCs was confirmed by western blotting (Supplemental Figure 1B). As shown in Figure 3, the changes in gene expression observed in response to deletion of *Gata4* in *Gata4*$^{flox/flox}$ pSCs were strikingly similar to those seen in *Gata4* siRNA treated TM4 cells, implying that TM4 cells are a reasonable model to study the consequences of GATA4 deficiency on SC function.

Notably, GATA4 depletion in TM4 cells and *Gata4*$^{flox/flox}$ pSCs was associated with dysregulation of genes involved the formation and remodeling of junctional complexes. Silencing of *Gata4* was accompanied by decreased expression of two tight junction genes: tight junction protein-1 (*Tjp1*) and claudin-12 (*Cldn12*) (Figure 3A,B). GATA4 depletion also led to aberrant expression of vinculin (*Vcl*), tenascin C (*Tnc*), and c-src tyrosine kinase (*Csk*) (Figure 3C-E), genes involved in formation of the apical ectoplasmic specialization (AES), a distinct actin-based adherens junction restricted to the Sertoli-spermatid interface (1, 34–36). Although not detected in the microarray, connexin 30.2 (*Cx30.2*), a component of gap junctions, was downregulated in GATA4-deficient TM4 cells and pSCs (Figure 3F). GATA4 depletion was associated with altered expression of genes encoding ECM proteins, proteases, and protease inhibitors implicated in the regulation of BTB remodeling and integrity. Among these were laminin 1 (*Lamc1*), type IV collagens (*Col4a1*, *Col4a5*), matrix metalloproteinase 10 (*Mmp10*), matrix metalloproteinase 23 (*Mmp23*), and tissue inhibitor of metalloproteinases 2 (*Timp2*) (Figure 3G-L). The gene en-

coding TNF, a cytokine released when ECM proteins are degraded (37), was upregulated in GATA4-deficient TM4 cells and SCs (Figure 3M). Collectively, these findings implicate GATA4 in the regulation of genes involved in BTB dynamics.

As further confirmation of our gene silencing models, we used qRT-PCR to examine the expression of certain other markers, including established targets of GATA4. In agreement with a recent report linking GATA4 to chemokine signaling in SCs (9), we found that silencing *Gata4* in TM4 cells and primary adult SCs led to decreased expression of the chemokines *Cxcl12 and Ccl9* (Figure 3N, O). Additionally, the mRNA levels of *Ccl25* and *Cxcl1* were altered following GATA4 depletion (Figure 3P, Q). Silencing of *Gata4* in TM4 cells and pSCs altered the expression of sex determining region Y-box 9 (*Sox9*) (Figure 3R), a transcription factor known to be regulated by GATA4/FOG2 (17). Krüppel family like protein 4 (*Klf4*), a transcription factor previously linked to claudin gene expression (19, 38), was also dysregulated in the knockdown cells (Figure 3S). Expression of another transcription factor gene, *Rhox5* (reproductive homeobox X-linked protein 5), an established SC marker (39), was not altered by GATA4 depletion in either TM4 cells or pSCs (Figure 3T).

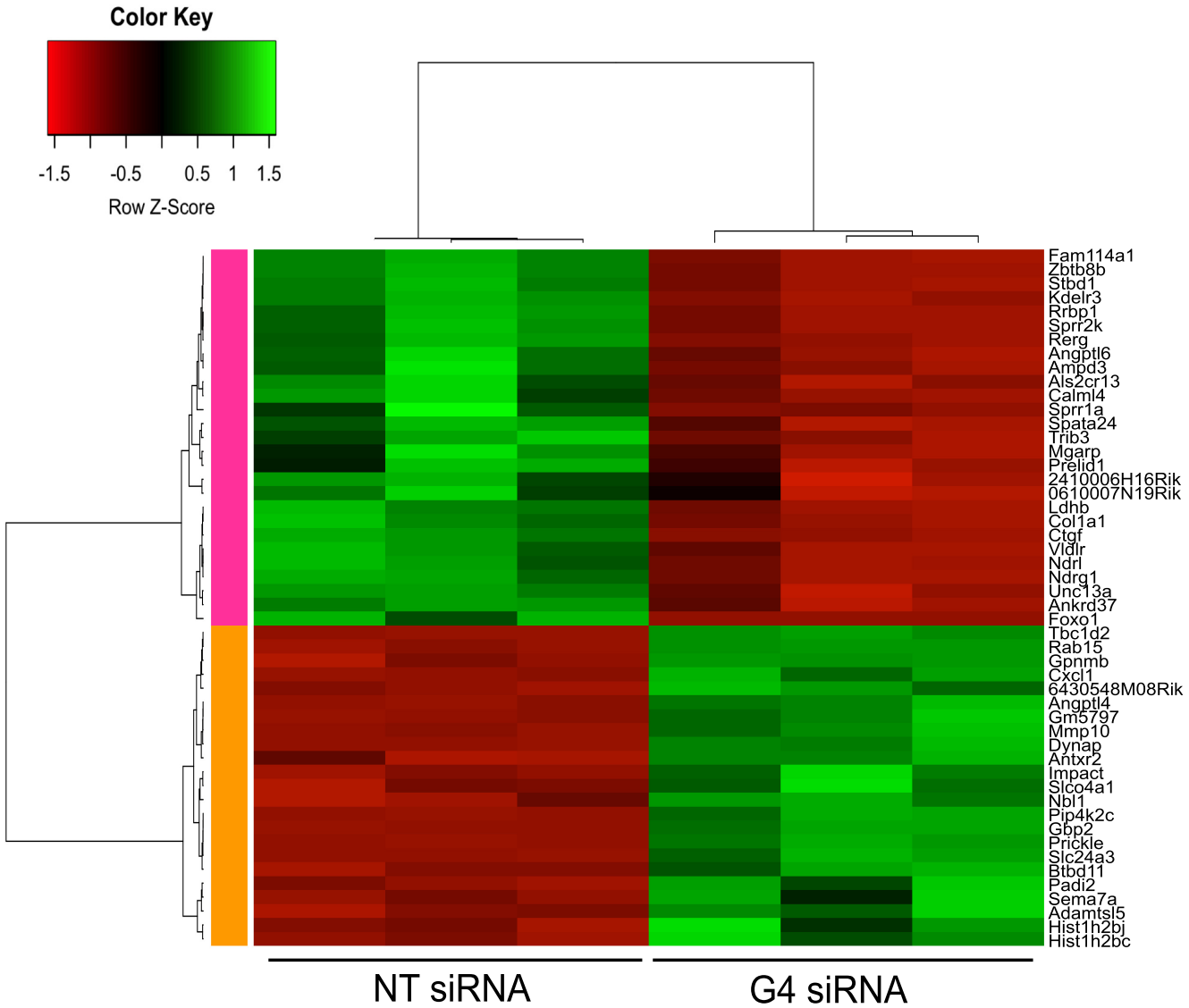

Figure 2. Gene expression profile of GATA4-deficient TM4 cells. Microarray analysis (n = 3 per group) was performed using the Illumina Mouse WG-6 v2.0 BeadChip. After background correction, quantile normalization, and log2 transformation, differentially expressed genes were identified using LIMMA with the Benjamini-Hochberg correction. Only probes with expression levels with at least 1.5-fold difference and a FDR below 5% were considered significantly differentially expressed. A heatmap showing the top 50 DEGs (sorted according to their log2 FCs) was generated with R. Red represents down-regulation and green signifies up-regulation of the particular probes.

Table 1. Gene set enrichment analysis of microarray data. Kyoto Encyclopedia of Genes and Genomes (KEGG) Gene Ontology (GO) pathway analysis results are arranged on the basis of *P* values. Size describes the overall number of genes related to one specific term, and # of genes represents the number of genes within this group that were significantly changed in microarray analysis ($n = 3$)

Analysis	Term	Size	# of genes	*P* value
KEGG Pathway	Focal adhesion	200	42	2.20E-06
	Metabolic pathways	1184	163	3.70E-06
	ECM- receptor interaction	86	19	7.10E-04
	D-Glutamine and D-glutamate metabolism	3	2	2.82E-02
	Adherens junction	75	13	3.50E-02
	Glycolysis / Gluconeogenesis	62	11	4.27E-02
Gene Ontology	Cadherin binding	23	2	4.00E-03
	Extracellular structure organization	161	4	4.60E-03
	L-Lactate-dehydrogenase activity	3	1	1.20E-02
	Lactate Metabolic Process	7	1	2.90E-02
	Protein localization to extracellular region	3	1	1.20E-02
	Extracellular region	1813	14	1.20E-02
	Proteinaceous extracellular matrix	333	5	1.10E-02

Decreased TJP1 protein levels in GATA4-deficient SCs

Spurred by the results of the transcriptomic analysis, we investigated the expression of *Tjp1*, a prototypical tight junction marker (40), in more detail. TM4 cells were transfected with *Gata4* siRNA or nontargeting siRNA and analyzed 72 hours later. Western blotting (Figure 4A) demonstrated a significant decrease (68 ± 23%) in the level of TJP1 protein in the GATA4-depleted cells (n = 4; $P < .01$), whereas the level of the housekeeping protein Actin was unchanged. Using the same method a slight reduction in TJP1 protein was also observed in *Gata4*$^{flox/flox}$ pSCs treated with cre-recombinase when compared to controls (Figure 4B). Immunofluorescence staining showed a concomitant decrease in GATA4 and TJP1 immunoreactivity in TM4 cells treated with *Gata4* siRNA vs. nontargeting siRNA (Figure 4C-F). Similarly, immunostaining of pSCs showed a loss of TJP1 protein from the cell surface in response to *Gata4* silencing (Figure 4G,H).

GATA4-depleted SCs cells exhibit impaired junctional complex formation and barrier function

To further probe the role of GATA4 in the formation of junctional complexes, TM4 cells were cultured on matrigel-coated slides, treated with *Gata4* siRNA or nontargeting siRNA, and then processed for EM (Figure 5A-D). Junctional complexes with the ultrastructural hallmarks of desmosomes (arrowheads, Figure 5C) were detected readily in cells treated with nontargeting siRNA but not in cells treated with *Gata4* siRNA (Figure 5D). The GATA4-depleted TM4 cells contained increased number of vacuoles (Figure 5B,D), a phenotypic feature previously reported in the SCs of *Gata4* conditional knockout mice generated with *Amhr2*-cre (9, 19).

To assess the consequences of GATA4 deficiency on epithelial barrier function, we measured transepithelial resistance (TER), an indicator of the paracellular barrier to ion conductance (31). For this analysis TM4 cells, WT pSCs, or *Gata4*$^{flox/flox}$ pSCs were grown as monolayers on matrigel-coated bicameral units and treated with siRNA or adenovirus (Figure 5E-G). Beginning 3 days after siRNA transfection, a significantly lower TER was observed in *Gata4* siRNA–treated TM4 cells than in TM4 cells treated with nontargeting siRNA (Figure 5E). *Gata4* silencing in pSCs was associated with a significantly lower TER at even earlier time points, ie, at day 2.5 in *Gata4*$^{flox/flox}$ pSCs subjected to adrenoviral-mediated cre-recombination (Figure 5F) or day 1.5 in WT pSC treated with siRNA (Figure 5G).

In theory, changes in cell viability or number could account for the observed differences in TER. We found that cell viability, measured with an MTS-based assay, was not significantly altered in either *Gata4* siRNA-treated TM4 cells (Supplemental Figure 2A) or GATA4-deficient *Gata4*$^{flox/flox}$ pSCs subjected to cre-mediated recombination (Supplemental Figure 2B). *Gata4* silencing led to significantly reduced cell numbers only after day 5 (TM4 cells) or day 6 (*Gata4*$^{flox/flox}$ pSCs and WT pSCs) (Supplemental Figure 2C-E). Thus, the early (day 1–4) differences in TER between GATA4-depleted SCs and controls cannot be attributed to reduced cell viability or number.

GATA4-depleted SCs exhibit aberrant expression of genes involved in lactate metabolism

The production of lactate via glycolysis and glutaminolysis, to fulfill the energy needs of developing spermatocytes and spermatids, is a crucial function of SCs (11). KEGG pathway and GO analyses of GATA4-depleted TM4 cells identified significant changes in pathways controlling lactate and glutamine metabolism (glycolysis, L-lactate-dehydrogenase activity, lactate metabolic process, as well as D-glutamine and D-glutamate metabolism) (Table 1). Subsequent qRT-PCR analysis of GATA4-depleted

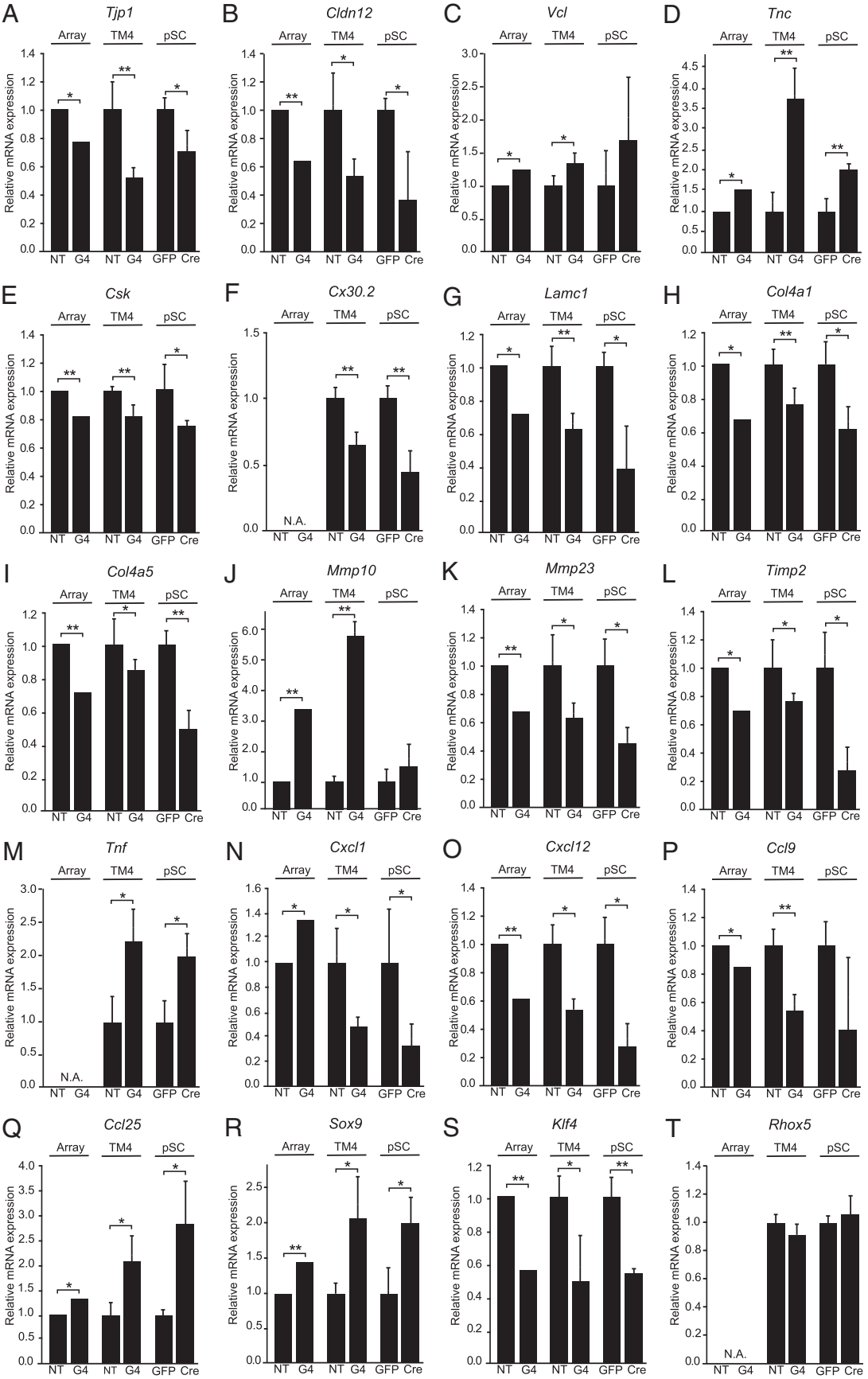

Figure 3. Impact of *Gata4* silencing on gene expression in TM4 cells and primary mouse Sertoli cells. Each panel shows the relative mRNA expression results for a specific gene, as determined by 3 different methods: (method 1, Array) microarray analysis of mRNA derived

TM4 and *Gata4*$^{flox/flox}$ pSCs confirmed the downregulation of glycolytic enzymes hexokinase 1 (*Hk1*), glucose phosphate isomerase 1 (*Gpi1*), phosphofructokinase (*Pfkp*), and phosphoglycerate mutase 1 (*Pgam1*) (Figure 6A-D).

Two other genes of importance in SC lactate metabolism, *Pdk3* and *Pkd4,* encode kinases that modulate the activity of pyruvate dehydrogenase complex (PDC), determining whether pyruvate is converted to lactate or alternatively oxidized via the TCA cycle (41–43). In SCs, the follicle stimulating hormone (FSH) induced upregulation of *Pdk3* with concomitant downregulation of *Pdk4* was recently shown to increase lactate production (44). Interestingly, depletion of GATA4 in TM4 and *Gata4*$^{flox/flox}$ pSCs elicited the reciprocal changes of decreased *Pdk3* and increased *Pdk4* expression (Figure 6E, F). Glutaminase 2 (*Gls2*), which catalyzes the first step in the conversion of glutamine to lactate via glutaminolysis, was downregulated in *Gata4* siRNA treated TM4 and *Gata4*$^{flox/flox}$ pSCs (Figure 6G). In addition, altered expression of LDH B (*Ldhb*) was also evident in the GATA4-deficient cells (Figure 6H). Taken together the above mentioned changes in gene expression suggest that GATA4 influences the production of lactate.

Metabolomic analysis of GATA4-depleted TM4 cells demonstrates impaired lactate production

The physiological consequences of treatment of TM4 cells with *Gata4* siRNA vs. nontargeting siRNA were determined by comprehensive metabolomic profiling using LC-MS/MS. Unsupervised principle component analysis demonstrated a separation of the sample set into 2 groups based on the metabolic profiles (Figure 7A), thus confirming that the metabolic profile of GATA4-depleted TM4 cells differed from that of control cells.

To identify the most significantly changed metabolites, we performed a partial least squares discriminant analysis followed by quantitative enrichment analysis (QEA). The top 20 changed metabolites are presented in Figure 7B. A heatmap of all the analyzed metabolites and a list of the pathways identified by QEA are shown in Supplemental Figure 3 and 4, respectively. The metabolite exhibiting the largest change was glutamine, which was enriched 2.3-fold in the GATA4-depleted TM4 cells (Figure 7B), suggesting an impaired conversion of this amino acid to lactate via glutaminolysis (10). Glutamine is known to inhibit the incorporation of alanine into protein (45), and indeed alanine levels were increased in the GATA4-deficient cells (Figure 7B). *Gata4* silencing also led to increased levels of several other amino acids (serine, glycine, threonine, asparagine, and aspartate) that, like alanine, normally function as alternative fuel sources in SCs (10, 45) (Figure 7B). The concentration of the metabolic waste product ammonium, a byproduct of glutaminolysis, was lower in conditioned media from GATA4 siRNA-treated TM4 cells and cre-treated *Gata4*$^{flox/flox}$ pSCs when compared to controls (Figure 7C).

In keeping with the aforementioned metabolic changes, the concentration of lactate was significantly lower in conditioned media from GATA4-deficient SCs as compared to controls (Figure 7D), whereas the concentration of glucose was significantly higher (TM4 cells) or unchanged (*Gata4*$^{flox/flox}$ pSCs) (Figure 7E).

Finally, LDH activity was measured in all three *Gata4* knockdown cell preparations (Figure 7 F,G). Reduced enzyme activity was evident in cells lacking GATA4.

Collectively, these findings are indicative of both an overall reduced metabolic activity and significantly impaired lactate production in the GATA4-depleted cells. Thus, silencing of *Gata4* in SCs cells was associated with a loss of the Warburg phenotype that normally typifies the metabolism of this cell type. Although there are known differences between the biology of immortalized SC cell lines and pSCs (46) the results obtained from the TM4 cell line and pSCs are strikingly similar, reinforcing the notion that TM4 cells are a useful model to study SC metabolism.

Discussion

Multiple lines of evidence support the notion that GATA4 plays a key role in the differentiation and function of SCs

Legend to Figure 3 Continued. . .
from TM4 cells 72 hours post siRNA treatment with nontargeting (NT) or *Gata4* (G4) siRNA (n = 3), (method 2, TM4) qRT-PCR analysis of mRNA derived from TM4 cells 72 hours post siRNA treatment with NT or G4 siRNA (n = 4), and (method 3, pSC) qRT-PCR analysis of mRNA derived from primary *Gata4*$^{flox/flox}$ Sertoli cells (*Gata4*$^{F/F}$ pSC) 48 hours post infection with adrenovirus expressing either cre + GFP (Cre) or GFP alone (GFP) (n = 4). Microarray results are presented as relative fold changes in mRNA expression. qRT-PCR results, normalized to *Actb* and ribosomal protein *L19* mRNA, are presented as relative expression values of the mean ± S.D. **, $P < .01$; * $P < .05$. *Tight junction associated genes:* (A) Tight junction protein 1 (*Tjp1*); (B) Claudin 12 (*Cldn12*). *AES associated genes:* (C) Vinculin (*Vcl*); (D) tenascin C (*Tnc*); (E) c-src tyrosine kinase (*Csk*); *Gap junction associated genes:* (F) Connexin 30.2 (*Cx30.2*). *Components of ECM:* (G) laminin, gamma 1 (*Lamc1*); (H) collagen, type IV, alpha 1 (*Col4a1*); (I) collagen, type IV, alpha 5 (*Col4a5*). *Metalloproteinases and protease inhibitors:* (J) matrix metalloproteinase 10 (*Mmp10*); (K) matrix metallopeptidase 23 (*Mmp23*); (L) tissue inhibitor of metalloproteinase 2 (*Timp2*). *Cytokine and chemokine signaling:* (M) tumor necrosis factor (TNF) (*Tnf*); (N) C-X-C motif chemokine 1 (*Cxcl1*); (O) C-X-C motif chemokine 12 (*Cxcl12*); (P) C-C motif ligand 9 (*Ccl9*); (Q) C-C motif ligand 25 (*Ccl25*). *Transcription factors:* (R) sex determining region Y-box 9 (*Sox9*); (S) Kruppel-like factor 4 (*Klf4*); (T) reproductive homeobox 5 (*Rhox5*). Note that *Cx30.2, Tnf*, and *Rhox5* were not represented on the microarray (N.A.= not available).

(17, 47). GATA4 is expressed in SCs throughout fetal and adult life (18, 48–56). Promoter analyses and related studies have identified groups of putative target genes for GATA4 in SCs, including genes involved in sex determination (*Sry*, *Sox9*, *Dmrt1*) (20, 57–60), FSH signaling (*Fshr*) (61, 62), cell-cell interactions (*Clmp*, *Cldn11*, *Cx30.2*) (19, 38, 63), and peptide hormone production (*Inha*, *Inhba*, *Amh*) (64). Studies of genetically-engineered mice demonstrate that GATA4 is required for early testicular development, germ cell licensing, maintenance of the SSC niche, and spermatogenesis (9, 19, 20, 58–60, 65, 66). Mouse fibroblasts can be efficiently reprogrammed into embryonic Sertoli-like cells using *Gata4* in combination with *Nr5a1*, *Wt1*, *Dmrt1*, and *Sox9* (67). Although genetic studies in the mouse provide strong evidence that GATA4 is essential for SC development and function, the molecular pathways regulated by this transcription factor have not been fully elucidated, especially in SCs of the adult animal. The results described herein provide new insights into the downstream targets of GATA4 in adult SCs. Specifically, our findings suggest that GATA4 plays a pivotal role in the regulation of BTB function and lactate metabolism in mouse SCs.

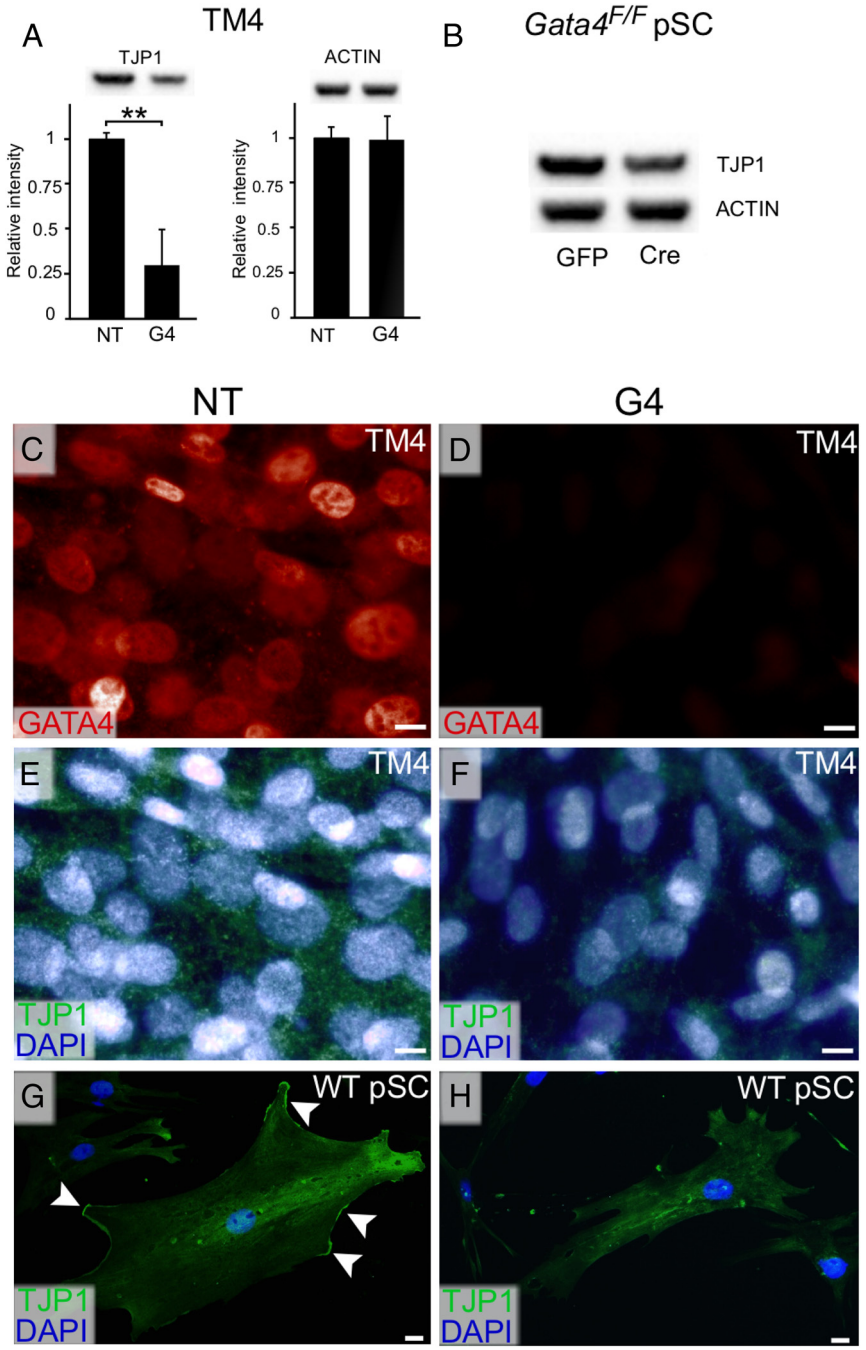

Figure 4. Reduced TJP1 protein levels in GATA4-depleted Sertoli cells. Cells were treated with either siRNA for 72 hours (A; C-H) or adenovirus for 48 hours (B) and grown in the absence of matrigel. A–B, Western blot analysis of tight junction protein 1 (TJP1) protein levels with ACTIN antibody as control. Quantitative protein signal was determined using Quantity One 1-D Analysis Software (Biorad, Hercules, CA). The data is presented as mean relative expression values ± S.D. (**, $P < .01$, n = 4). C–H, TM4 cells and wild-type primary Sertoli cells (WT pSC) were fixed in 4% PFA and subjected to double immunofluorescence staining. GATA4 (red) and TJP1 (green); DAPI (blue) was used as nuclear stain. Note the disappearance of TJP1 protein from the cell surface after GATA4 loss in WT pSCs. Bars = 2 μm.

Silencing of *Gata4* in TM4 cells and pSCs was associated with decreased expression of the tight junction genes *Tjp1* and *Cldn12*. *Tjp1* encodes phosphoprotein that localizes to the cytoplasmic membrane surface at sites of cell-cell contact (68), and *Cldn12* encodes a transmembrane protein implicated in barrier function (69). The downregulation of *Tjp1* and *Cldn12* in GATA4-depleted TM4 cells was accompanied by a loss of morphologically recognizable junctional complexes. TM4 cells and pSCs deficient for GATA4 further showed a decline in transepithelial membrane resistance. Prior studies have implicated GATA factors in the regulation of other genes important for BTB integrity, including the tight junction associated genes *Cldn2* (70) and *Cldn11* (63).

Cx30.2, encoding a gap junction protein hypothesized to mediate interactions between SCs and germ

cells (71), was downregulated in GATA4-deficient TM4 cells and pSCs. *Cx30.2* is a known target of GATA4 in the heart (72), and decreased expression of *Cx30.2* has been reported in whole testis extracts from conditional knockout mice lacking GATA4 in adult SCs (19).

GATA4 depletion in TM4 cells and pSCs also led to dysregulated expression of genes involved in the AES (*Vcl*, *Tnc*, *Csk*). One of the functions of the AES is to prevent the release of immature spermatozoa into the lumen of the seminiferous epithelium (34). Interestingly, mice harboring a conditional deletion of *Gata4* in SCs exhibit premature release of spermatocytes and spermatids into the tubule lumen (19).

As reviewed in detail elsewhere (5), SC-derived ECM proteins have been shown to regulate tight junction remodeling during spermatogenesis, and genes encoding basement membrane components (*Lamc1*, *Col4a1*, *Col4a5*) were downregulated in GATA4-depleted TM4

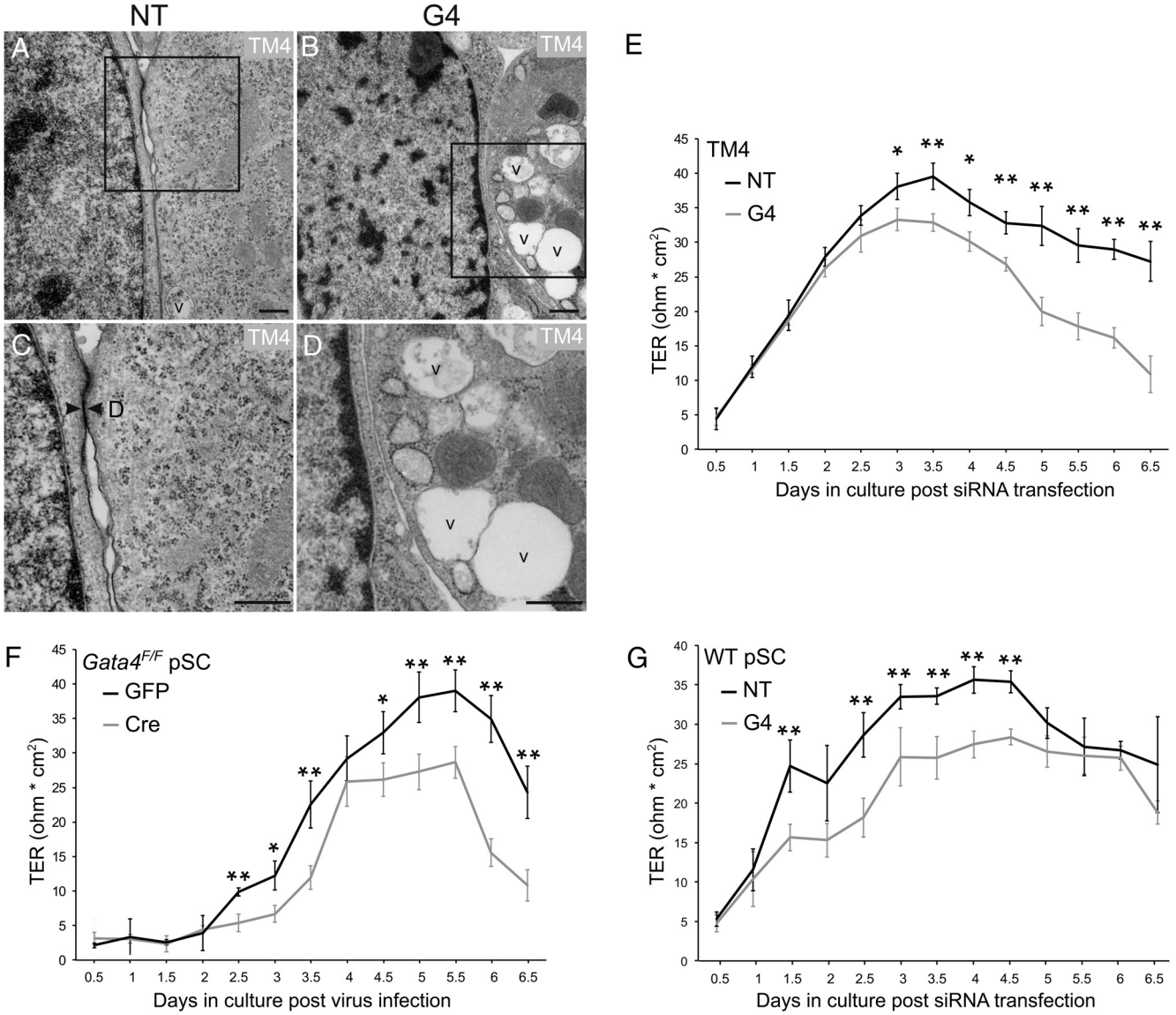

Figure 5. Decreased junctional complexes and epithelial barrier resistance in GATA4-depleted SCs. A–D, Shown are representative electron micrographs of TM4 cells grown on matrigel coated chamber slides. Cells were transfected with *Gata4* siRNA (G4) or nontargeting siRNA (NT), and then processed for EM 72 hours later. Higher magnification views of the boxed areas in panels A & B are shown in panels C & D, respectively. Note the presence of large vacuoles in the GATA4-deficient cells (B, D). Even though two adjacent cells are aligned over a long distance and their membranes are juxtaposed, junctional complexes with ultrastructural hallmarks of desmosomes are absent in GATA4-depleted cells (B,D). No morphological features of apoptosis, such as homogenous chromatin condensation within nuclei, were evident after GATA4 depletion. Abbreviations: D, Desmosome; v, vacuole. Bars, 500 nm. E–G, Indicated cells were either transfected with siRNA or infected with adenovirus (gray line = *Gata4* knockdown cells; black line = control cells) and cultured at a density of 0.5×10^6 cells/cm^2 (TM4) or 1.2×10^6 cells/cm^2 (*Gata4*$^{F/F}$ and WT pSC) in matrigel coated bicameral culture units. The establishment of a tight junction permeability barrier was assessed by measurement of transepithelial resistance (TER). Values are expressed as the mean ± S.D. (*, $P < .05$; **, $P < .01$; n = 3).

cells and pSCs. Providing independent evidence that GATA4 impacts the expression of ECM genes, decreased levels of laminin and type IV collagen have been reported in embryoid bodies derived from *Gata4*$^{-/-}$ embryonic stem cells (73).

Metalloproteinases, protease inhibitors, and cytokines are known to impact BTB integrity and remodeling (5, 74). *Mmp10* was upregulated in GATA4-deficient TM4 cells and pSCs, whereas *Mmp23* and *Timp2* were downregulated. These findings are consistent with a prior report showing that tissue-remodeling genes, including *Mmp23*, are dysregulated in the ovaries of *Gata4/6* double conditional knockout mice (75).

Taken together, these marked derangements in the expression profile of genes important for BTB integrity and SC–ECM interactions underline the crucial role of GATA4 for physiological SC function. One additional upregulated factor in GATA4-depleted SCs is TNF, a cytokine released when ECM proteins are degraded (37). Earlier studies revealed that TNF perturbs SC tight junction formation in a dose-dependent manner and that *Gata4* conditional knockout mice, suffering from a leaky BTB, have elevated TNF mRNA levels (19, 76). Interestingly, TNF is known to stimulate lactate production in SCs (77), suggesting a link between deranged ECM-SC interactions and lactate production in these cells.

The profound metabolic changes observed in GATA4-depleted TM4 cells are summarized in Figure 8 and a probable attempt to maintain lactate production. Glutamine, the most elevated metabolite in GATA4-depleted TM4 cells, is one of the most important substrates for the production of lactate. Indeed, conversion of this amino acid to lactate via glutaminolysis has been reported to yield much of the energy required by SCs (13, 45). The decreased expression of *Gls2* in GATA4-deficient TM4 cells and pSCs offers a plausible explanation for the elevation of intracellular glutamine levels. Glutamine is known to inhibit the incorporation of alanine during protein anabo-

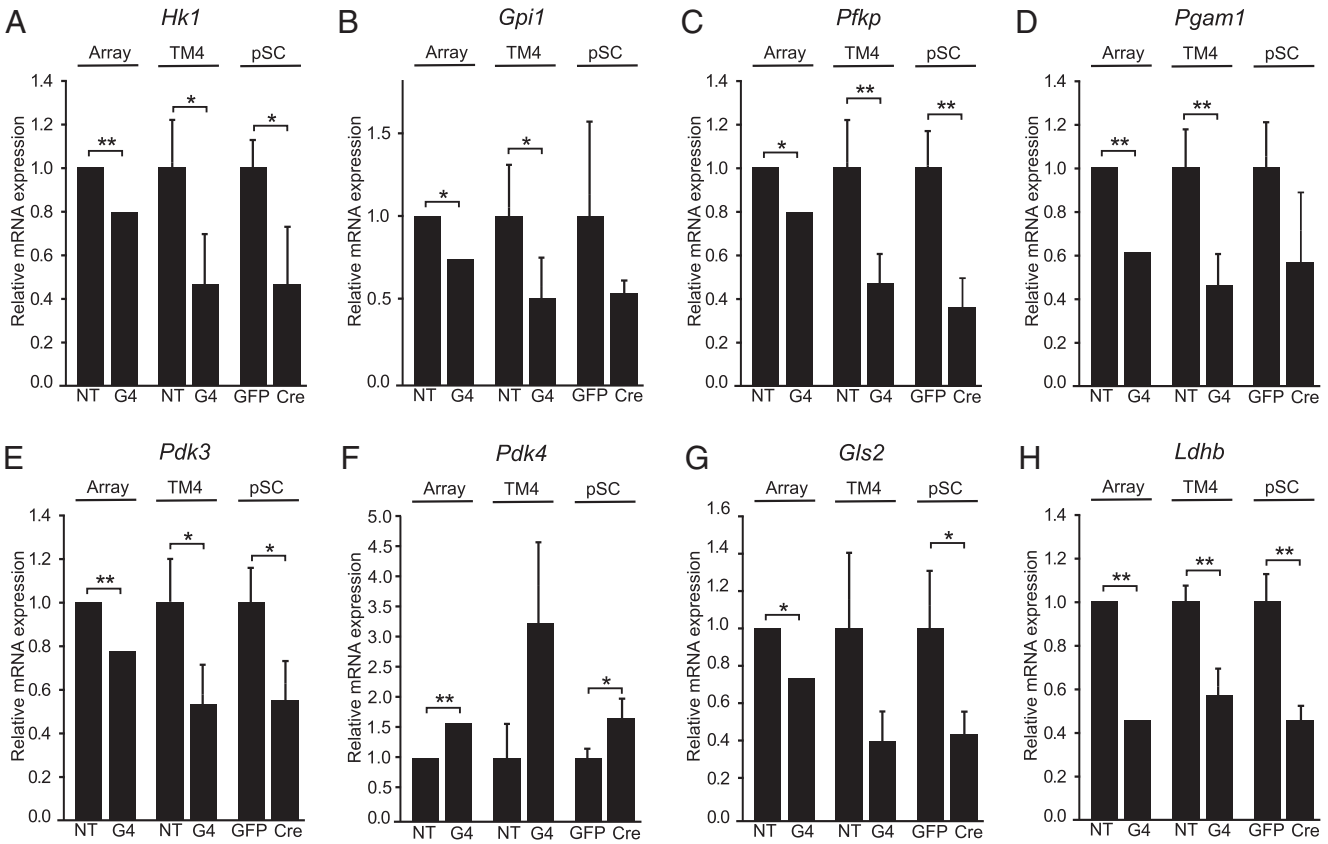

Figure 6. Altered expression of genes impacting lactate metabolism in GATA4-depleted SCs. A–H, Each panel shows the relative mRNA expression results for a specific gene, as determined by 3 different methods: (method 1, Array) microarray analysis of mRNA derived from TM4 cells 72 hours post siRNA treatment with nontargeting (NT) or *Gata4* (G4) siRNA (n = 3), (method 2, TM4) qRT-PCR analysis of mRNA derived from TM4 cells 72 hours post siRNA treatment with NT or G4 siRNA (n = 4), and (method 3, pSC) qRT-PCR analysis of mRNA derived from primary *Gata4*$^{flox/flox}$ Sertoli cells (*Gata4*$^{F/F}$ pSC) 48 hours post infection with adrenovirus expressing either cre + GFP (Cre) or GFP alone (GFP) (n = 4). Microarray results are presented as relative fold changes in mRNA expression. qRT-PCR results, normalized to *Actb* and ribosomal protein *L19* mRNA, are presented as relative expression values of the mean ± S.D. **, $P < .01$; * $P < .05$. A, hexokinase 1 (*Hk1*); (B) glucose phosphate isomerase 1 (*Gpi1*); (C) phosphofructokinase, platelet (*Pfkp*); (D) phosphoglycerate mutase 1 (*Pgam1*); (E) pyruvate dehydrogenase kinase, isoenzyme 3 (*Pdk3*); (F) pyruvate dehydrogenase kinase, isoenzyme 4 (*Pdk4*); (G) glutaminase 2 (*Gls2*), (H) lactate dehydrogenase (LDH) B (*Ldhb*).

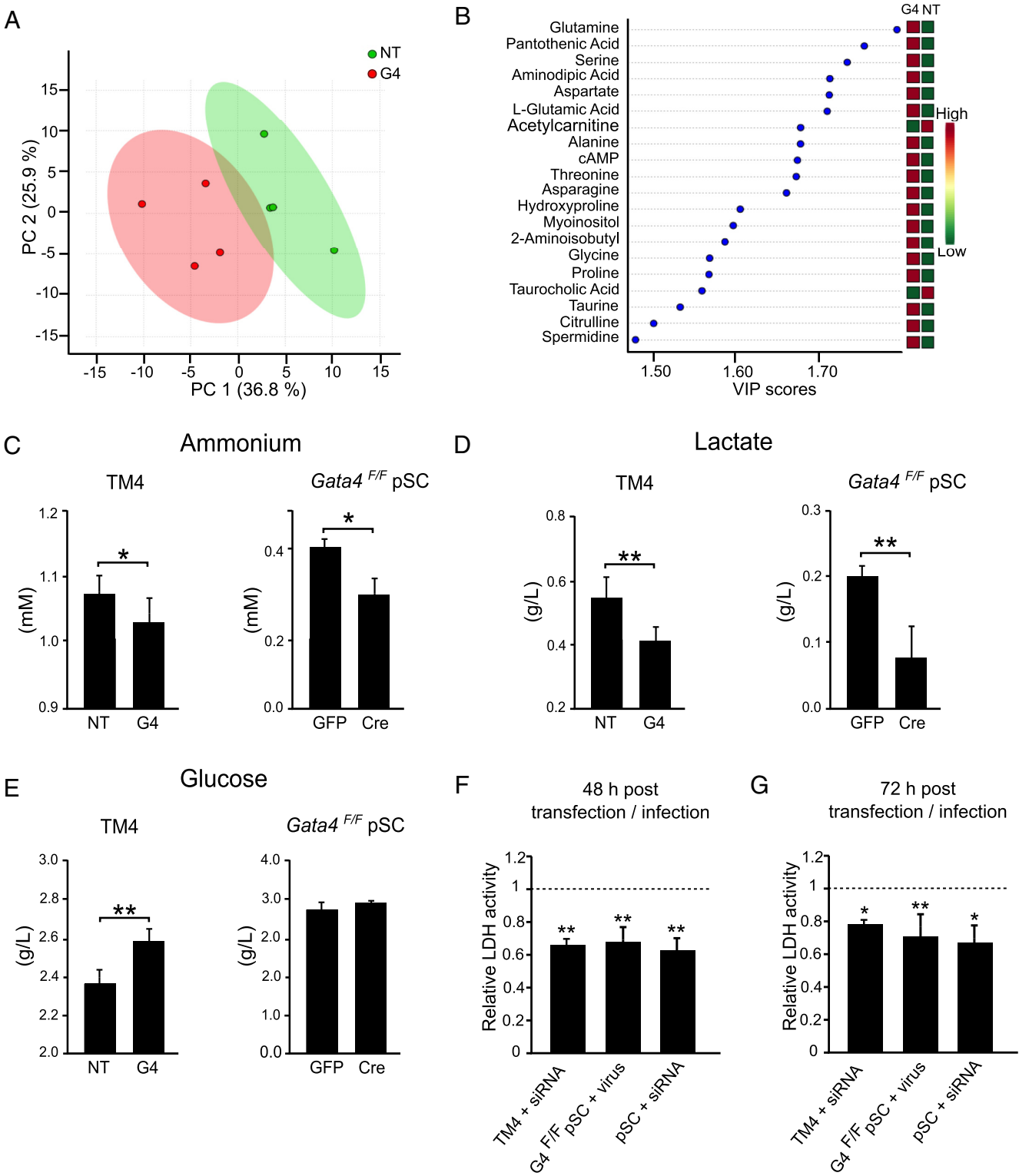

Figure 7. Altered metabolic profile and lactate metabolism in GATA4-depleted SCs. (A, B) For metabolic profiling of TM4 cells a total of 110 metabolites were analyzed 72 hours post siRNA transfection using mass spectrometry (n = 4). A, Principle component (PC) analysis revealed the unsupervised separation between of the 2 sample groups [*Gata4* (G4) siRNA vs. nontargeting (NT) siRNA treated] based on differences in their metabolic profiles. B, Partial least squares discriminant analysis identified the 20 most significantly changed metabolites and their relative abundance in control and GATA4-depleted cells. The components were sorted according to the variable of importance in the projection (VIP) for the first component. Relative magnitudes of each metabolite disturbance are listed on the right as high (red) and low (green). C–E, Concentrations of ammonium, lactate, and glucose in conditioned media from TM4 cells and *Gata4*$^{flox/flox}$ pSCs (*Gata4*$^{F/F}$ pSC) were quantified with Konelab Arena 20 XT analyzer (n = 4–7) 72 hours post siRNA transfection or 48 hours post infection with adenovirus, respectively. (F,G) 48 hours and 72 hours post transfection or infection cells of all three *Gata4* knockdown experiments were trypsinized and counted; 5000

lism (45). This is of importance in SCs since alanine can be converted into pyruvate, thus serving as a substrate to maintain lactate production. GATA4-deficient TM4 cells accumulated other amino acids (serine, threonine, asparagine, glycine, proline, aspartate) normally used as alternative fuel sources to support lactate production (10). These changes are indicative of an overall reduced metabolic activity in TM4 cells lacking GATA4. Consistent with this notion of reduced metabolic activity, conditioned media from GATA4-depleted TM4 cells and pSCs contained decreased concentrations of lactate and the metabolic waste product ammonium.

The three major steps in shunting glucose to lactate within SCs are regulated by glycolytic enzymes (converting glucose to pyruvate), PDC (regulating the entry of pyruvate into the TCA cycle), and LDH (converting pyruvate to lactate) (Figure 8A). The glycolytic activity of GATA4-depleted SCs was attenuated as evidenced by reduced expression of key glycolytic genes (*Hk1*, *Gpi1*, *Pfkp*, *Pgam1*) and attenuated glucose utilization. This is in accordance with our recent findings in Leydig cells, which exhibit diminished glycolytic activity in response to GATA4 depletion (24).

PDC activity is tightly regulated through a reversible phosphorylation/dephosphorylation mechanism (41). Phosphorylation of PDC by PDK isoforms leads to an inactive state of PDC, whereas dephosphorylation by pyruvate dehydrogenase phosphatase (PDP) leads to an active state. Consequently, the relative activities of PDK and PDP determine the flux of pyruvate into the TCA cycle (42, 43). A recent study showed that FSH increases lactate production in rodent SCs by regulating this balance; following FSH exposure, *Pdk3* mRNA levels increased, while *Pdk4* mRNA levels decreased (44). We observed the opposite effect ($\downarrow$ *Pdk3*, $\uparrow$ *Pdk4*) in GATA4-depleted pSCs and an attendant decrease in lactate production. Hence, *Gata4* silencing in SCs mimics the phenotypic changes associated with FSH withdrawal. GATA4-deficient TM4 cells have elevated intracellular levels of succinate (Supplemental Figure 4), consistent with increased flow of pyruvate into the TCA cycle.

The final step of lactate production, the conversion of pyruvate to lactate (Figure 8), is catalyzed by LDH, a tetramer composed of LDHA and/or LDHB subunits. The random combination of these subunits into tetramers results in 5 LDH isoenzymes: LDH-1 (B4), –2 (A1 B3), –3

Legend to Figure 7 Continued. . .
cells of each sample were lysed and LDH activity was determined. Results are presented as fold variation to control. C–G, Values are expressed as the mean ± S.D. (*, $P < .05$; **, $P < .01$).

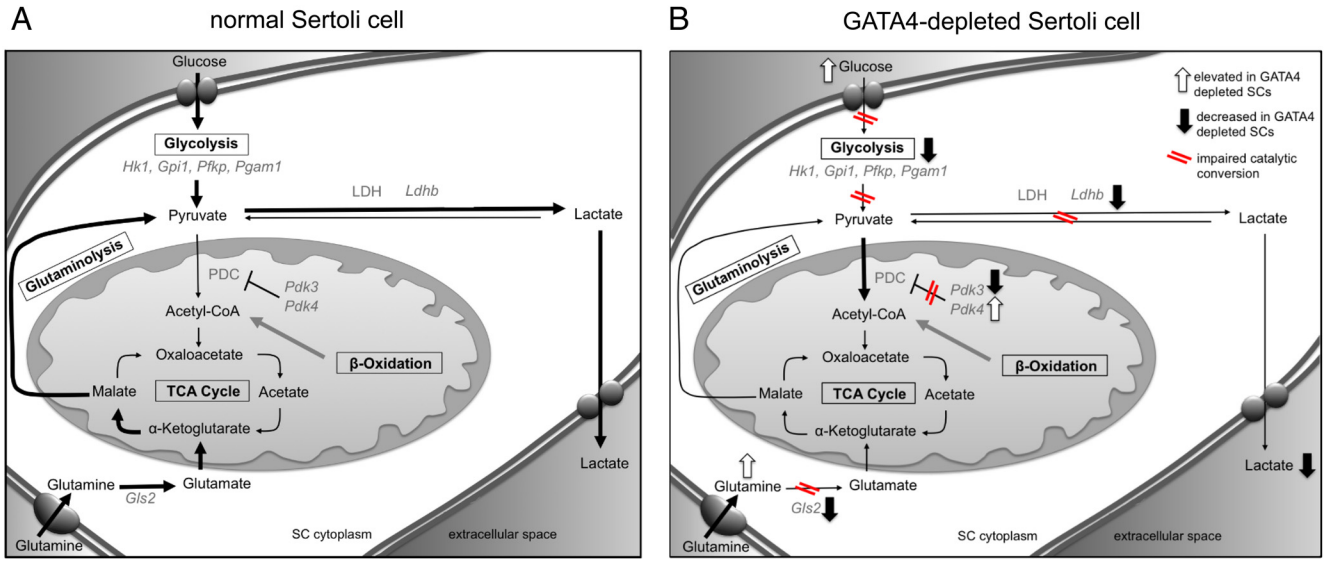

Figure 8. Impact of GATA4 depletion on lactate metabolism in SCs. Shown are metabolic pathways important for production of lactate, the principal source of energy for postmeiotic germ cells. A, Normal SCs exhibit a Warburg phenotype and use glucose and glutamine for the production of lactate. Key reactions in shunting glucose to lactate within SCs are catalyzed by LDH and pyruvate dehydrogenase complex (PDC). PDC activity is inhibited through phosphorylation by pyruvate dehydrogenase kinase 3 and 4 (PDK3 and PDK4). Normally, only 25% of the pyruvate generated through glycolysis is oxidized via the TCA cycle (13) and is instead converted into lactate, which then is secreted (14). Glutaminase 2 (GLS2) converts glutamine into glutamate, which can be further metabolized to lactate via glutaminolysis. B, SCs lacking GATA4 no longer exhibit the Warburg-like state, as evidenced by changes in mRNA levels (Italic letters) or metabolite concentrations. The most important alterations identified in this study are indicated with white (elevated) or black (decreased) arrows, and impaired catalytic steps following GATA4 depletion are highlighted in red. The figure is adapted from Oliveira et al, 2015 (10).

(A2 B2), –4 (A3 B1), and –5 (A4) (78). Recent reports suggest that LDHA may be the most important LDH subunit in SCs (15), although LDHB is also present in this cell type (78, 79). We observed reduced *Ldhb* expression accompanied with a decreased LDH activity in GATA4-depleted TM4 cells and pSCs.

SC-derived lactate is the principal energy source for mature germ cells, and lactate has a stimulating effect on RNA and protein synthesis in spermatids (80). Impaired lactate production in SCs can have profound effects on spermatogenesis. Concentrations of lactate are low in the testes of the cryptorchid rat, and intratesticular infusion of lactate into these animals improves spermatogenesis (81). In addition to serving as an energy source, lactate functions as an antiapoptotic factor in mature testicular germ cells (82). We surmise that the apoptosis of haploid germ cells seen in *Gata4* conditional knockout mice generated with *Amhr2*-cre (19) reflects in part an insufficient supply of SC-derived lactate.

In summary, we propose that GATA4 functions physiologically to regulate the integrity of blood-testis barrier and apical ectoplasmic specializations and to maintain SCs in a Warburg-like state, thereby ensuring a robust supply of lactate for nourishment of germ cells. GATA4 exerts its metabolic effects by transmitting signals of the FSH pathway and regulating the expression and activity of key enzymes involved in lactate production. The concept that transcription factor GATA4 can govern intracellular metabolism was recently demonstrated in another testicular somatic cell type - the Leydig cell (24, 83, 84). Manipulation of *Gata4* expression in SCs affords a means to study the Warburg effect in a noncancerous cell type. These findings come on the heels of a report demonstrating that GATA4 plays a central role in senescence; in a variety of cell types the DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4 (85). Thus, GATA4 has emerged as a pivotal regulator of a diverse array of biological processes, including stem cell maintenance, cell-cell interactions, intermediary metabolism, inflammation, and senescence.

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GATA4 Is a Key Regulator of Steroidogenesis and Glycolysis in Mouse Leydig Cells

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Transcription factor GATA4 is expressed in somatic cells of the mammalian testis. Gene targeting studies in mice have shown that GATA4 is essential for proper differentiation and function of Sertoli cells. The role of GATA4 in Leydig cell development, however, remains controversial, because targeted mutagenesis experiments in mice have not shown a consistent phenotype, possibly due to context-dependent effects or compensatory responses. We therefore undertook a reductionist approach to study the function of GATA4 in Leydig cells. Using microarray analysis and quantitative RT-PCR, we identified a set of genes that are down-regulated or up-regulated after small interfering RNA (siRNA)-mediated silencing of *Gata4* in the murine Leydig tumor cell line mLTC-1. These same genes were dysregulated when primary cultures of *Gata4*$^{flox/flox}$ adult Leydig cells were subjected to adenovirus-mediated cre-lox recombination in vitro. Among the down-regulated genes were enzymes of the androgen biosynthetic pathway (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*, and *Srd5a*). Silencing of *Gata4* expression in mLTC-1 cells was accompanied by reduced production of sex steroid precursors, as documented by mass spectrometric analysis. Comprehensive metabolomic analysis of GATA4-deficient mLTC-1 cells showed alteration of other metabolic pathways, notably glycolysis. GATA4-depleted mLTC-1 cells had reduced expression of glycolytic genes (*Hk1*, *Gpi1*, *Pfkp*, and *Pgam1*), lower intracellular levels of ATP, and increased extracellular levels of glucose. Our findings suggest that GATA4 plays a pivotal role in Leydig cell function and provide novel insights into metabolic regulation in this cell type. **(*Endocrinology* 156: 1860–1872, 2015)**

Transcription factor GATA4 has been implicated in the differentiation and function of cells in the mammalian testis (1, 2). During fetal testicular development, *Gata4* is expressed in pre-Sertoli cells, Sertoli cells, fetal Leydig cells, fibroblast-like interstitial cells, and peritubular myoid cells (3–5). In the adult testis, GATA4 is expressed in Sertoli cells, Leydig cells, and stem Leydig cells (6–12). Promoter analyses and related studies have identified sev-

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Abbreviations: Ad, adenovirus; Ad-cre-IRES-GFP, Ad expressing the combination of cre recombinase and GFP; Ad-GFP, Ad expressing GFP; CREB, cAMP-responsive element binding protein; DCA, dichloroacetic acid; 2DG, 2-deoxy-D-glucose; FC, fold change; FDR, false discovery rate; FOG, friend of Gata; GFP, green fluorescent protein; GO, Gene Ontology; *Gpi1*, glucose phosphate isomerase 1; *Hk1*, hexokinase 1; HSD, hydroxy steroid dehydrogenase; IRES, internal ribosome entry site; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NAD, nicotinamide adenine dinucleotide; *Pfkp*, phosphofructokinase; *Pgam1*, phosphoglyceratemutase 1; PKA, protein kinase A; qRT-PCR, quantitative RT-PCR; RHOX5, reproductive homeobox 5; siRNA, small interfering RNA.

eral groups of putative target genes for GATA4 in testis, including genes associated with sex determination (*Sry*, *Sox9*, and *Dmrt1*) (13–17), peptide hormone production (*Inha*, *Inhba*, and *Amh*) (18), gonadotropin signaling (*Fshr* and *Lhcgr*) (19, 20), steroid synthesis (*Star*, *Cyp11a1*, and *Cyp17a1*) (14, 21–24), and cell-cell interactions (*Clmp*, *Cldn11*, and *Cx30.2*) (25–27).

Gata4 knockout mice die by embryonic day 9.5 due to defects in ventral morphogenesis and heart development (28, 29), so the role of this transcription factor in gonadal function cannot be determined from these animals. Analysis of other genetically engineered mice has shown that interactions between GATA4 and its cofactor, friend of Gata 2 (FOG2 or ZFPM2), regulate early testis development (14–16). $Fog2^{-/-}$ mice and $Gata4^{ki/ki}$ mice, which bear a knock-in mutation that abrogates the interaction of GATA4 with FOG cofactors (30), exhibit similar testicular phenotypes, including decreased testicular *Sry* expression, aberrant differentiation of early Sertoli cells, and sex reversal (14, 16). More recently, conditional mutagenesis studies have established that GATA4 is required for genital ridge development, expression of *Dmrt1* gene in fetal Sertoli cells, testis cord morphogenesis, and adult Sertoli cell function (17, 25, 31). Collectively, these studies establish that GATA4 plays an essential role in the differentiation and maintenance of Sertoli cells in the fetal and adult mouse.

The role of GATA4 in Leydig cell development, however, remains controversial, because gene targeting experiments in mice have not shown a consistent phenotype (reviewed in Ref. 2). For example, $Gata4^{-/-}$ progenitors exhibit an impaired capacity to differentiate into fetal Leydig cells in the testis of chimeric mice (2, 4). On the other hand, conditional mutagenesis of *Gata4* in Leydig cells as early as embryonic day 12.5 does not cause an overt impairment in the expression of Leydig cell differentiation markers in the fetal or adult testis (2, 17).

Interpreting the results of targeted mutagenesis experiments in the mouse testis is challenging because of context-dependent effects, variable degrees of cre-mediated recombination, compensatory responses, alternative pathways of differentiation, and functional redundancy (2). To circumvent these limitations, we have assessed the impact of *Gata4* deficiency on Leydig cell function in 2 less complicated experimental models: an immortalized mouse Leydig tumor cell line (mLTC-1) and primary cultures of adult mouse Leydig cells. Using an integrated approach, including transcriptome and metabolome analyses, we show that *Gata4* deficiency has profound effects on specific metabolic pathways, especially steroidogenesis and glycolysis.

Materials and Methods

Animals and cultured cells

Experiments involving mice were approved by the institutional committee for laboratory animal care at Washington University. $Gata4^{flox/flox}$ mice (also termed $Gata4^{tm1.1Sad}$/J) were purchased from The Jackson Laboratory and genotyped as described (32, 33). Murine primary Leydig cells were isolated from 3- to 6-month-old animals using Percoll density separation (34). Primary cells were maintained in DMEM/F12+GlutaMAX media supplemented with 10% fetal bovine serum, 25mM HEPES, and 100-mg/L penicillin/streptomycin (all from Life Technologies). Leydig cell preparations were determined to be 90%–95% pure on the basis of immunostaining for 3β-hydroxy steroid dehydrogenase/isomerase (3βHSD) and the Sertoli cell marker reproductive homeobox5 (RHOX). mLTC-1 cells were originally purchased from Rebois and Griswold (35) and cultured in Waymouth's medium supplemented with 5% fetal bovine serum, 10% horse serum, and 100-mg/L gentamicin (all from Life Technologies).

Knockdown of *Gata4* in mLTC-1 cells and primary adult Leydig cells

mLTC-1 cells (passages 10–16) were transiently transfected in the absence of antibiotics with a pool of 4 small interfering RNAs (siRNAs) targeting *Gata4* (5′-AGAGAAUAGCUUCGAACCA-3′, 5′-GGAUAUGGGUGUUCCGGGU-3′, 5′-CUGAAUAAAU-CUAAGACGC-3′, 5′-GGACAUAAUCACCGCGUAA-3′) or with nontargeting control siRNA (5′-UGGUUUACAUGUCGAC-UAA-3′; all from Dharmacon) using Lipofectamine RNAiMAX transfection reagent in Opti-MEM (Life Technologies) at a final concentration of 0.1μM. Conditioned media and cells were collected 72 hours after transfection for the analyses described below. Primary Leydig cells were cultured in the presence of adenovirus (Ad) (multiplicity of infection, 100) expressing either green fluorescent protein (GFP) (Ad-GFP) or the combination of cre recombinase and GFP [Ad-cre-internal ribosome entry site-GFP (Ad-cre-IRES-GFP)] (Vector Biolabs). After infection, the cells were maintained in serum-free DMEM/F12+GlutaMAX (Life Technologies) for 24 hours before RNA extraction.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using the Nucleospin RNA/Protein kit (Machrey-Nagel) and reverse transcribed using SuperScript VILO cDNA Synthesis kit (Life Technologies). qRT-PCR was performed using SYBR GREEN I (Invitrogen), and expression was normalized to the housekeeping gene *Actb*. Primer pairs are listed in Supplemental Table 1.

Western blotting

Protein was extracted from cell cultures with the NucleoSpin RNA/Protein kit (Machrey-Nagel), and 20 μg of protein was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Invitrogen). A list of antibodies used is provided in Supplemental Table 2. Immun-Star WesternC kit (Bio-Rad) was used for detection.

Immunocytochemistry

mLTC-1 cells and primary cells were grown on 8-well glass Lab Tek Chamber Slides (Sigma) and fixed 72 hours after trans-

fection or 24 hours after infection with 4% paraformaldehyde in PBS. Immunoperoxidase and indirect immunofluorescent staining were performed as described (36). See Supplemental Table 2 for a list of antibodies.

Microarray expression profiling and gene set enrichment analysis

RNA was isolated from mLTC-1 cells 72 hours after transfection with *Gata4* or nontargeting siRNA (n = 3) using NucleoSpin RNA/Protein kit and purified with NucleoSpin RNA Clean-up XS kit (both from Machrey-Nagel). RNA quality was assessed via Bioanalyzer (Agilent). Array hybridization was performed by the Functional Genomics Unit at the University of Helsinki using an Illumina MouseWG-6 v2.0 oligonucleotide BeadChip. Data were background corrected using BeadStudio software (Illumina); quantile normalization and log2 transformation were performed using the BeadArray bioconductor package (37). Differentially expressed genes were identified using linear models for microarray data (38) with Benjamini-Hochberg correction. Expression levels with at least 1.5× difference and a false discovery rate (FDR) below 5% were considered as significantly differentially expressed. Microarray data were subjected to average linkage clustering with uncentered correlation using Cluster (39). A heatmap was generated with R. Gene set enrichment analysis of the differentially expressed genes was performed using GOstats bioconductor package (40). Hypergeometric tests with the Benjamini-Hochberg FDR were performed to adjust the *P* value.

Cell viability and apoptosis assays

Cell viability was assayed using CellTiter 96 Aqueous One Solution (Promega). Caspase 3/7 activation was measured using Caspase-Glo 3/7 Assays (Promega). Absolute luminescence was normalized using values obtained from wells containing nontransfected mLTC-1 cells.

Steroid hormone measurements

Conditioned media samples from mLTC-1 cells were used to measure steroid levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (41). For steroid separation and quantification, an Agilent 1200 Series HPLC system connected to a mass spectrometer (Agilent 6410 Triple Quadrupole LC/MS) was used.

Metabolite measurements

Metabolites were extracted from cell samples, separated using Acquity UPLC and analyzed using XEVO TQ-S Triple Quadrupole LC/MS (Waters Corp). At 72 hours after transfection, approximately 2 million mLTC-1 cells per sample were washed with PBS and deionized water and subsequently quenched in liquid nitrogen. Metabolites were extracted by adding 20 μL of labeled internal standard mix and 1 ml of cold extraction solvent (80/20 acetonitrile/H_2O + 1% formic acid). Extracts were vacuum filtered (δ pressure 300–400 mbar for 2.5 min; Hamilton) and injected into the LC system. A detailed description of instrument parameters is given elsewhere (42). A total of 96 metabolite concentrations were measured and data were normalized and analyzed using MetaboAnalyst 2.0 software.

ATP assay

At varying time points after siRNA treatment (24, 48, or 72 h) mLTC-1 cells were exposed to 20mM 2-deoxy-D-glucose (2DG) (Caymen Chemical Co) for 4 hours, followed by 1 hour incubation with 20mM dichloroacetic acid (DCA) (Sigma). Cellular ATP signal levels were measured with ATPlite Luminescence Assay System (PerkinElmer).

Quantification of glucose, lactate, and ammonium concentrations

Conditioned cell culture media of mLTC-1 cells was collected 72 hours after transfection, and glucose, lactate, and ammonium concentrations were measured with Konelab Arena 20 XT (Thermo Electron Oy) as previously described (43).

Statistical methods

mRNA levels, absorbance values for viability assay, luminescence intensities, and steroid levels were analyzed using the Student's *t* test or when appropriate, one-way ANOVA followed by Dunnett's test. Statistical significance was set at *, $P < .05$ and **, $P < .01$.

Results

Changes in gene expression associated with knockdown of *Gata4* in mLTC-1 cells

We used siRNA to inhibit *Gata4* expression in mLTC-1 cells. To evaluate the efficiency of gene silencing, RNA and protein were isolated from mLTC-1 cells 72 hours after siRNA transfection. *Gata4* mRNA levels were reduced by 80 ± 8% in cells treated with *Gata4* siRNA vs nontargeting siRNA-treated cells (n = 3; $P < .01$) (Figure 1). Western blotting showed no residual GATA4 protein band in the *Gata4* siRNA-treated cells (Figure 1B), and immunocytochemistry confirmed markedly reduced GATA4 staining in the nuclei of the targeted cells (Figure 1, C and D).

Microarray hybridization analysis was used to assess the impact of *Gata4* silencing on the mLTC-1 transcriptome. A total of 3495 probes were differentially expressed (1740 up-regulated, 1755 down-regulated). Results were ranked according to their log2 fold change (FC) values. Unsupervised hierarchical clustering of the top 50 differentially expressed probes is shown in Figure 2, and FC values for a subset of genes of interest are listed in Table 1. Complete microarray hybridization results are available via Gene Expression Omnibus accession number GSE63320.

Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed with all of the 3495 differentially expressed probes to identify biological processes and pathways affected by the inhibition of *Gata4* in mLTC-1 cells (Table

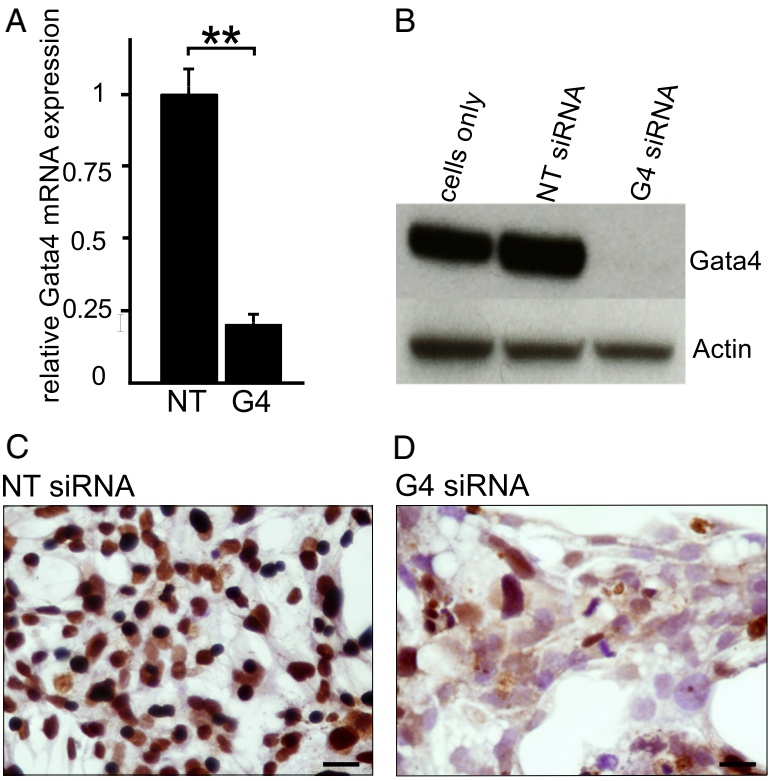

Figure 1. siRNA mediated inhibition of *Gata4* expression in mLTC-1 cells. Cells were treated with nontargeting (NT) siRNA or *Gata4* (G4) siRNA for 72 hours. The efficiency of gene silencing was determined using qRT-PCR, Western blotting, and immunocytochemistry. A, qRT-PCR results, normalized to *Actb* mRNA, are presented as mean relative expression values ± SD (**, $P < .01$, n = 4). B, Western blot analysis with β-actin was as control. C and D, GATA4 immunoperoxidase staining of mLTC-1 cells exposed to NT siRNA and *Gata4* siRNA, respectively. Cells were counterstained with hematoxylin. Scale bars, 15 μm.

2). Among the terms identified in the GO analysis were steroid biosynthetic process, sex differentiation, regulation of endocrine process, and response to hormone stimulus, all of which reflect the main function of Leydig cells, biosynthesis of steroids. Other terms identified in the GO analysis were negative regulation of cell proliferation, regulation of cell death, and regulation of apoptotic process. KEGG analysis highlighted alterations in metabolic pathways such as pyruvate metabolism, fatty acid metabolism, and glycolysis/gluconeogenesis (Table 2). The putative disruptions in hormone production and cellular metabolism are explored in detail below.

To validate the microarray results, qRT-PCR analysis was performed on RNA isolated from mLTC-1 cells transfected with *Gata4* siRNA vs nontargeting siRNA (Figure 3). Significantly decreased expression levels were observed in genes implicated in Leydig cell function, including steroidogenic factors (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Srd5a*, *Nr5a1* [Sf1/AdBP4], *Lhcgr*, and *Star*) (Figure 3, A–G), peptide hormones (*Inha* and *Inhba*) (Figure 3, H and I), a gonadotropin-regulated protease inhibitor (*Serpina5*) (Figure 3J), a defensin expressed in gonads (*Defb19*) (Figure 3K), a pseudokinase of the Tribbles family (*Trib2*) (Figure 3L), and the growth factor receptor and proto-oncogene *Kit* (44–49) (Figure 3M). Decreases in the protein levels of HSD3B1 and Cytochrome P450 17A1 were confirmed by double immunofluorescence staining (Supplemental Figure 1, A and B) and Western blotting (Supplemental Figure 1C). Indolethylamine N-methyltransferase (*Inmt*), a gene known to be up-regulated in *Wwox*$^{-/-}$ mice with impaired steroidogenesis (50), was found to be up-regulated in GATA4-deficient mLTC-1 cells (Figure 3N). Likewise, prostaglandin D2 synthase (*Ptgds*), a gene implicated in Leydig cell function (51), was confirmed to be up-regulated in the *Gata4* siRNA-treated cells (Figure 3O). Importantly, expression of *Insl3*, which encodes insulin-like 3, a hormone constitutively secreted by Leydig cells, was comparable in the knockdown and control mLTC-1 cells (Figure 3P). This lack of change in the expression of Insl3, an established serum marker of Leydig cell biomass and function (52), suggests that the observed changes in expression of other genes do not reflect cytotoxicity (52).

Changes in gene expression associated with knockdown of *Gata4* in cultured primary adult Leydig cells

Immortalized mLTC-1 cells do not fully recapitulate the behavior of all endogenous Leydig cell populations. For example, mLTC-1 cells do not express *Hsd17b3*, the final enzyme in the testosterone biosynthetic pathway, and consequently do not secrete this sex steroid (53, 54). In this regard, the steroidogenic capacity of mLTC-1 cells more closely resembles that of fetal rather than adult Leydig cells (55). We therefore assessed the impact on *Gata4* inhibition on primary cultures of adult Leydig cells. Rather than relying on siRNA, we inhibited *Gata4* expression in primary Leydig cells via cre-mediated recombination; primary Leydig cells isolated from *Gata4*$^{flox/flox}$ mice were infected in vitro with the cre-expressing adenoviral vector Ad-cre-IRES-GFP or the control vector Ad-GFP. Based on GFP expression, the infection efficiency of the adenoviral vectors was estimated to be approximately 95% each. qRT-PCR analysis showed that infection of *Gata4*$^{flox/flox}$ primary Leydig cells with Ad-cre-IRES-GFP vs Ad-GFP resulted in 50 ± 7% inhibition of *Gata4* at 48 hours after

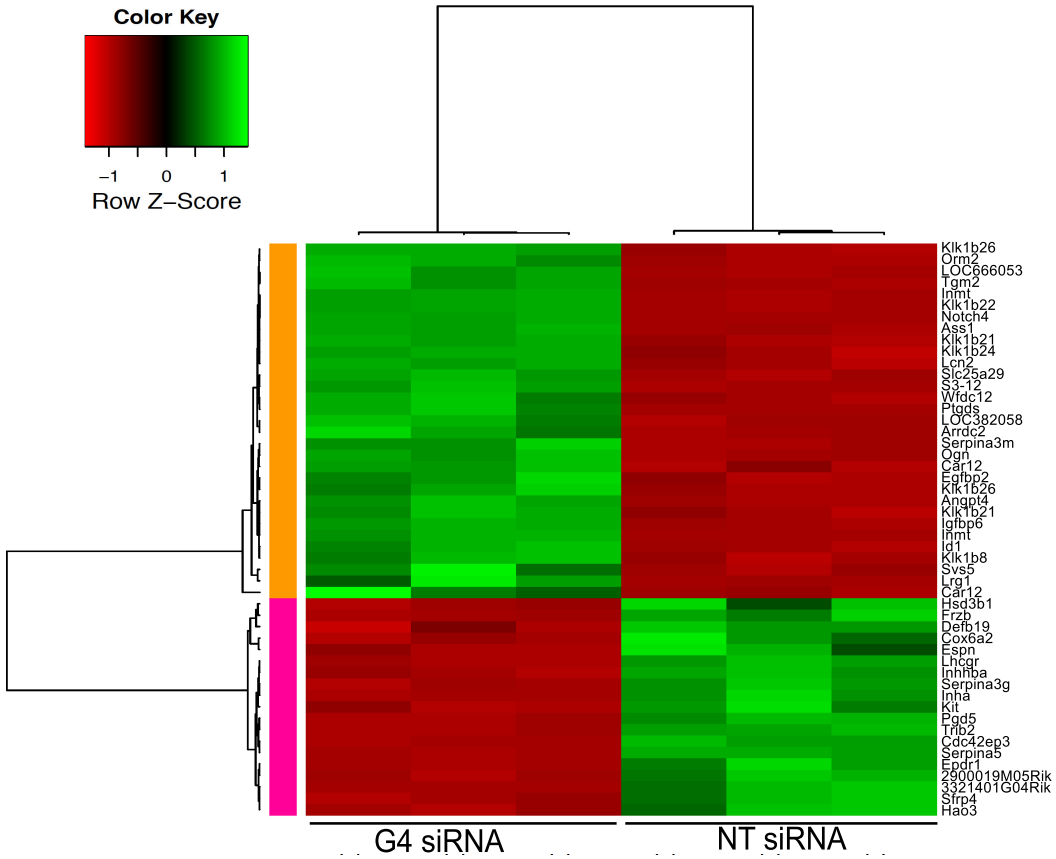

Figure 2. Gene expression profile of GATA4-deficient mLTC-1 cells. Microarray analysis (n = 3) was performed using the Illumina Mouse WG-6 v2.0 BeadChip. After background correction, quantile normalization, and log2 transformation, differentially expressed genes were identified using LIMMA with the Benjamini-Hochberg correction. Only probes with expression levels with at least 1.5-fold difference and a FDR below 5% were considered significantly differentially expressed. A heatmap showing the top 50 DEGs (sorted according to their log2 FCs [log FCs]) was generated with R. Red represents down-regulation and green signifies up-regulation of the particular probes.

infection (n = 4; $P < .01$). The changes in gene expression observed in response to knockdown of *Gata4* in primary Leydig cells were strikingly similar to those seen in *Gata4* siRNA-treated mLTC-1 cells (Figure 3). These similarities suggest that: 1) the specific metabolic effects associated with GATA4 depletion in mLTC-1 cells (see below) are not merely an artifact of the siRNA transfection system, and 2) mLTC-1 cells are a reasonable model to study the consequences of GATA4 deficiency on Leydig cell function. We therefore focused the ensuing analysis on mLTC-1 cells, because these cells are easier to maintain and experimentally manipulate than primary Leydig cells.

Knockdown of *Gata4* in mLTC-1 cells is associated with decreased production of sex-steroid precursors

Leydig cells synthetize testosterone from cholesterol in a pathway catalyzed by series of enzymes (Figure 4). We used LC-MS/MS to quantify sex steroid precursors in conditioned media from mLTC-1 cells treated with *Gata4* siRNA vs nontargeting siRNA. Compared with control cells, media from GATA4-depleted mLTC-1 cells contained significantly lower concentrations of pregnenolone and the Δ^5-pathway metabolites progesterone, androstenedione, and androstanedione (n = 3; $P < .01$) (Figure 4B). Notably, the level of androstenedione, the immediate progenitor of testosterone, was reduced markedly in the knockdown cells (*Gata4* siRNA: 24.2 ± 4.8nM vs nontargeting siRNA: 172.7 ± 48.1nM, n = 3; $P < .01$) (Figure 4B). We conclude that the down-regulation of steroidogenic gene expression in *Gata4* siRNA-treated mLTC-1 cells (Figure 3, A–D) is accompanied by a concomitant decrease in the production of sex steroid precursors via the Δ^5-pathway, the preferred route for androgen biosynthesis in mLTC-1 cells (54).

GATA4 deficiency alters the metabolic profile of mLTC-1 cells

Next, we used LC-MS/MS to assess the impact of GATA4 deficiency on other metabolic pathways. Unsupervised principle component analysis demonstrated a separation of the sample set into 2 groups based on metabolic profiles (Figure 5), confirming that the metabolic

Table 1. Microarray Expression Values for a Subset of Genes of Interest

Gene	FC	Adj. *P* Value
Serpina5	0.16	3.70E-09
Trib2	0.19	3.10E-09
Inha	0.19	6.10E-08
Kit	0.28	5.20E-06
Inhba	0.32	7.60E-07
Hsd3b1	0.33	1.30E-05
Defb19	0.34	5.00E-05
Lhcgr	0.34	2.50E-07
Cyp17a1	0.42	3.70E-06
Srd5a1	0.49	5.80E-06
Cyp11a1	0.50	8.80E-06
Gata4	0.50	3.20E-05
Ldha	0.55	6.00E-08
Igfbp5	0.56	7.70E-05
Hk1	0.66	3.40E-04
Pgam1	0.67	2.40E-06
Nr5a1	0.70	7.70E-04
Star	0.71	4.20E-04
Pdha1	0.77	8.20E-03
Gpi1	0.79	3.60E-04
Pfkp	0.86	3.30E-03
Gata6	1.25	2.20E-03
Igfbp6	3.05	1.10E-07
Inmt	8.37	2.50E-09
Ptgds	11.85	1.10E-08

The table lists differentially expressed genes (DEGs) according to their FCs between the 2 sample groups (mLTC-1 cells transfected with *Gata4* siRNA and nontargeting siRNA; n = 3) and adjusted *P* values.

profile of GATA4-depleted mLTC-1 cells differs from that of control cells.

To identify the most significantly changed metabolites, we performed partial least squares discriminant analysis (Figure 5B). Among the top 20 most significantly altered metabolites was nicotinamide adenine dinucleotide (NAD), a key regulator of redox reactions during glycolysis. NAD levels in *Gata4* siRNA-treated cells were decreased by 62 ± 4% (n = 3; $P < .01$) as compared with levels in control cells. Note that all the 20 most significant altered metabolites were decreased in GATA4-depleted cells, indicating an overall reduced metabolic activity. Relative differences in the levels of all measured metabolites are illustrated in a heatmap (Supplemental Figure 2).

Quantitative enrichment analysis identified biologically meaningful patterns at pathway levels based on the metabolomics data (Figure 5C). A total of 25 pathways were significantly altered (n = 3; $P < .05$) between the 2 sample groups. Based on the combination of *P* value and fold enrichment, glycolysis was identified to be the top hit among all of altered pathways ($P < .01$; FC = 4.3).

In keeping with decreased metabolic activity of the GATA4-depleted cells, the concentration of the metabolic waste product ammonium in conditioned media was significantly lower 72 hours after transfection (*Gata4* siRNA: 1.20 ± 0.03mM vs nontargeting siRNA: 1.39 ± 0.01mM, n = 8; $P < .01$) (Figure 5D).

In light of the GO term analysis and the aforementioned metabolic derangements, we assessed the impact of *Gata4* silencing on viability and survival of mLTC-1 cells (Supplemental Figure 3). Using a MTS-based assay, we found modest decreases in "cell viability" at time points of 48, 72, and 96 hours after transfection (n = 4; $P < .05$ for all time points) (Supplemental Figure 3A). These changes could reflect decreased cell proliferation or a reduced pro-

Table 2. Gene Set Enrichment Analysis of Microarray Data

Analysis	Term	Size	Number of Genes	*P* Value
GO	Steroid biosynthetic process	132	5	1.9 E-04
	Sex differentiation	212	6	2.1 E-04
	Regulation of endocrine process	32	3	2.9 E-04
	Reproductive system development	234	6	3.7 E-04
	Proteolysis	740	10	4.9 E-04
	Response to hormone stimulus	403	7	1.2 E-03
	Negative regulation of cell proliferation	472	7	2.9 E-03
	Regulation of cell death	1115	11	5.1 E-03
	Regulation of apoptotic process	1047	9	2.5 E-02
KEGG pathway	Terpenoid backbone biosynthesis	14	9	1.1 E-05
	Pyruvate metabolism	43	16	5.2 E-05
	Fatty acid metabolism	48	15	8.0 E-04
	Valine, leucine and isoleucine degradation	50	15	1.3 E-03
	Fructose and mannose metabolism	37	12	1.8 E-03
	Propanoate metabolism	33	10	7.4 E-03
	Glycolysis/gluconeogenesis	62	15	1.2 E-02
	Glyoxylate and dicarboxylate metabolism	19	6	1.9 E-02
	Alanine, aspartate, and glutamate metabolism	33	9	2.2 E-02
	Arginine and proline metabolism	54	12	4.2 E-02

GO and KEGG pathway analysis results are arranged on the basis *P* values. Size describes the overall number of genes related to 1 specific term, and number of genes is the number of genes significant changed in microarray analysis within this group.

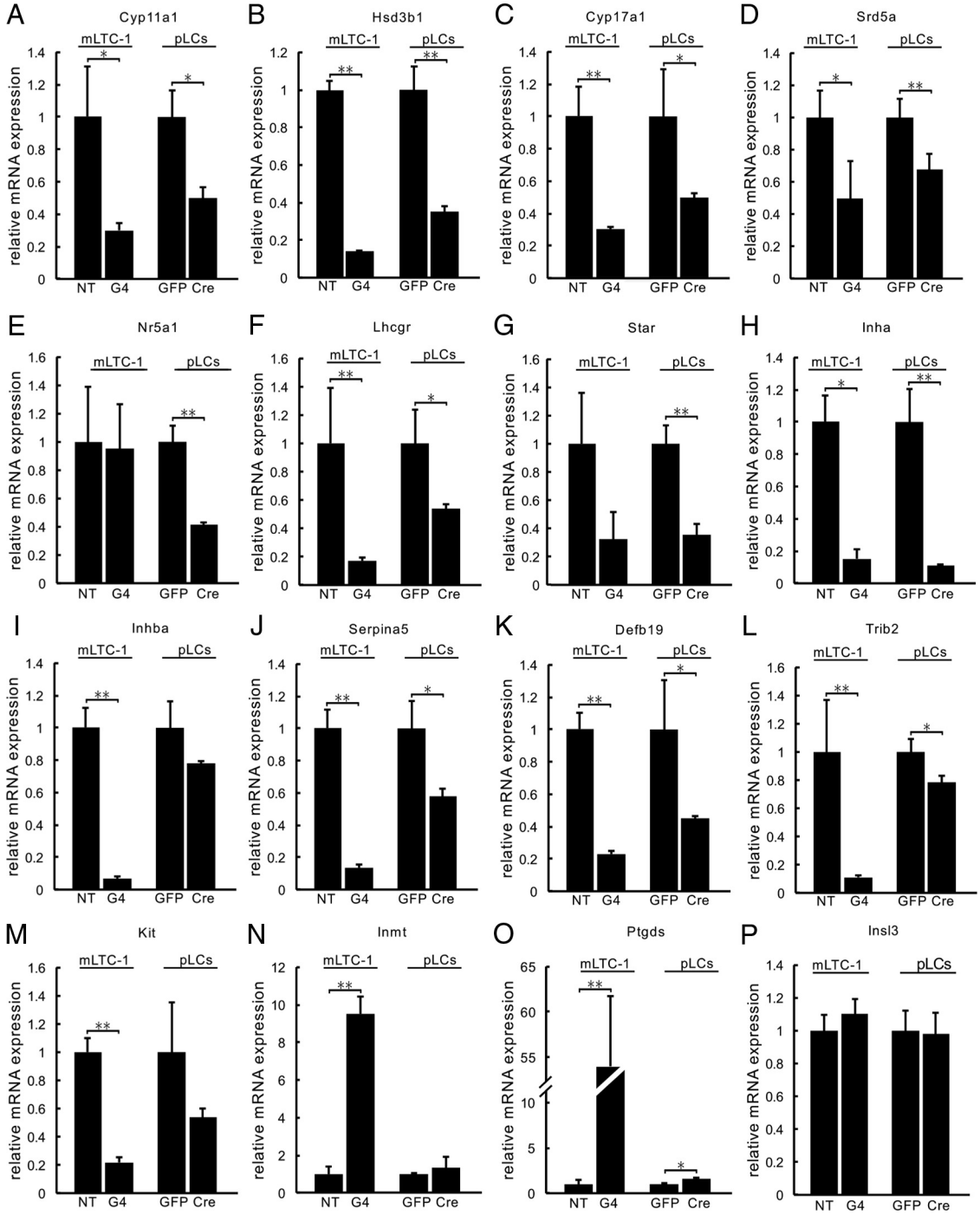

Figure 3. Knockdown of *Gata4* alters steroidogenic gene expression in mLTC-1 cells and primary mouse Leydig cells. qRT-PCR analysis of mRNA derived from mLTC-1 cells 72 hours after siRNA treatment and mRNA derived from primary *Gata4*$^{flox/flox}$ Leydig cells 24 hours after infection with Ad expressing Cre. Results, normalized to *Actb* mRNA, are presented as relative expression values of the mean ± SD (**, $P < .01$; *, $P < .05$, n = 3–4). pLCs, primary Leydig cells; NT, nontargeting siRNA; G4, *Gata4* siRNA; GFP, control Ad-GFP; Cre, Ad-Cre recombinase + GFP.

duction of reducing equivalents such as NADH or NADPH. Apoptosis of mLTC-1 cells was assayed 72 hours after siRNA transfection by measuring caspase 3/7 activity with a luminescence assay. *Gata4* knockdown in mLTC-1 cells increased caspase 3/7 activity by 27 ± 12% when compared with control cells transfected with nontargeting siRNA (n = 4; $P < .01$) (Supplemental Figure 3B).

GATA4 deficiency inhibits glycolysis in mLTC-1 cells

Consistent with the LC-MS/MS and microarray hybridization results qRT-PCR analyses showed significantly reduced expression of the glycolytic genes hexokinase 1 (*Hk1*), glucose phosphate isomerase 1 (*Gpi1*), phosphofructokinase (*Pfkp*), and phosphoglyceratemutase 1 (*Pgam1*) (Figure 6, A–D).

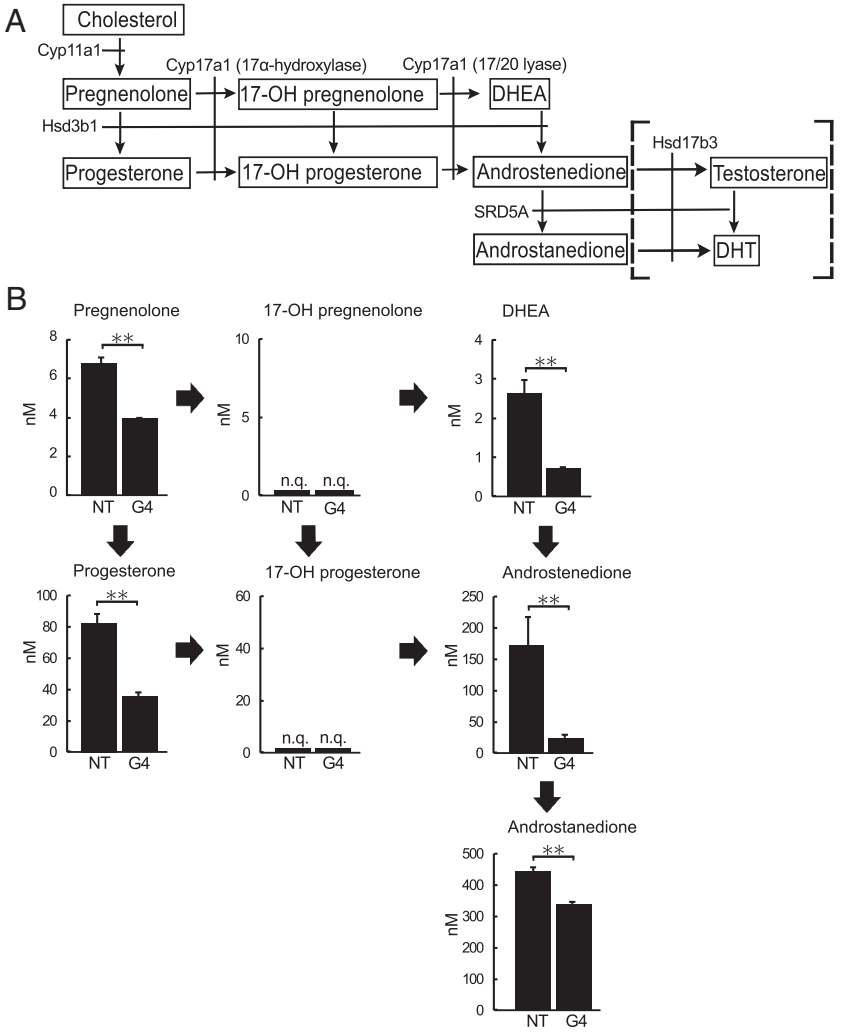

Figure 4. Decreased production of sex steroid precursors in GATA4-depleted mLTC-1 cells. A, Steroid metabolic pathway illustrating the conversion from cholesterol to testosterone. Pregnenolone, 17-OH pregnenolone, and dehydroepiandrosterone (DHEA) are Δ^4-steroid metabolites (Δ^4-pathway), whereas progesterone, 17-OH progesterone, and androstenedione are Δ^5-steroid metabolites (Δ^5-pathway). HSD3B1 functions as a $\Delta^{5/4}$ isomerase. B, Steroid metabolite concentrations in conditioned media from mLTC-1 cells were measured using LC-MS/MS 72 hours after siRNA transfection. Levels of 17-OH pregnenolone were below the assay detection limits, and concentrations of 17-OH progesterone were not quantifiable due to a double-peak appearance. Values are expressed as the mean ± SD (**, $P < .01$; n = 3). NT, nontargeting siRNA; G4, *Gata4* siRNA; n.q., not quantifiable.

Given this evidence of impaired glycolysis, we predicted that the amount of intracellular ATP would be decreased in *Gata4* siRNA-treated cells. Indeed, treatment of mLCT-1 cells with *Gata4* siRNA was associated with a 30 ± 12% reduction in intracellular ATP levels compared with nontargeted siRNA control cells (Figure 6E). By inhibiting glycolysis 72 hours after siRNA transfection with 2DG and/or DCA, we verified that differences in ATP levels between GATA4-depleted and control cells were predominantly due to effects of gene silencing on glycolysis. In nontargeting siRNA control cells, treatment with DCA or the combination of 2DG and DCA decreased ATP amounts significantly by 45 ± 16% and 54 ± 20%, respectively (n = 4; $P < .05$). By contrast, in *Gata4* siRNA-treated cells, ATP levels were minimally impacted by the addition of 2DG or DCA. Similar results were obtained at 48 hours after transfection, when GATA4 mRNA levels were reduced by 60 ± 5% (n = 4; $P < .05$) in *Gata4* siRNA-treated cells (Supplemental Figure 4B). Supposedly due to statistically not significantly changed GATA4 mRNA levels at 24 hours after transfection, no differences in the response to the addition of glycolytic inhibitors could be observed between the 2 sample groups (Supplemental Figure 4A).

Consistent with decreased glycolysis, glucose concentrations in conditioned media from *Gata4* siRNA-treated cells were significantly higher (*Gata4* siRNA: 3.57 ± 0.043 g/L vs nontargeting siRNA: 3.28 ± 0.025 g/L, n = 8; $P < .001$) (Figure 6F), and lactate concentrations were significantly lower (Gata4 siRNA: 0.77 ± 0.01 g/L vs nontargeting siRNA: 1.02 ± 0.025 g/L, n = 8; $P < .001$) (Figure 6G).

Discussion

Previous studies have provided circumstantial evidence that GATA4 is involved in Leydig cell function. *Gata4* is expressed in fetal and adult Leydig cells and in putative stem Leydig cells (3–12). Based on in vitro promoter analyses, this transcription factor has been implicated in the regulation of many genes that are expressed in Leydig cells (reviewed in Ref. 1). Conditional mutagenesis studies have established a role for GATA4 in the control of sex steroidogenic cell function in the mouse ovary (56–58). Gonadectomy of certain mouse strains triggers the ectopic expression of *Gata4* and other Leydig cell differentiation markers (eg, *Insl3* and *Cyp17a1*) in the adrenal cortex (59).

Although there is abundant indirect evidence implicating GATA4 in Leydig cell function, there is a dearth of direct (genetic) evidence. A missense mutation in the human *GATA4* gene has been linked abnormal testicular

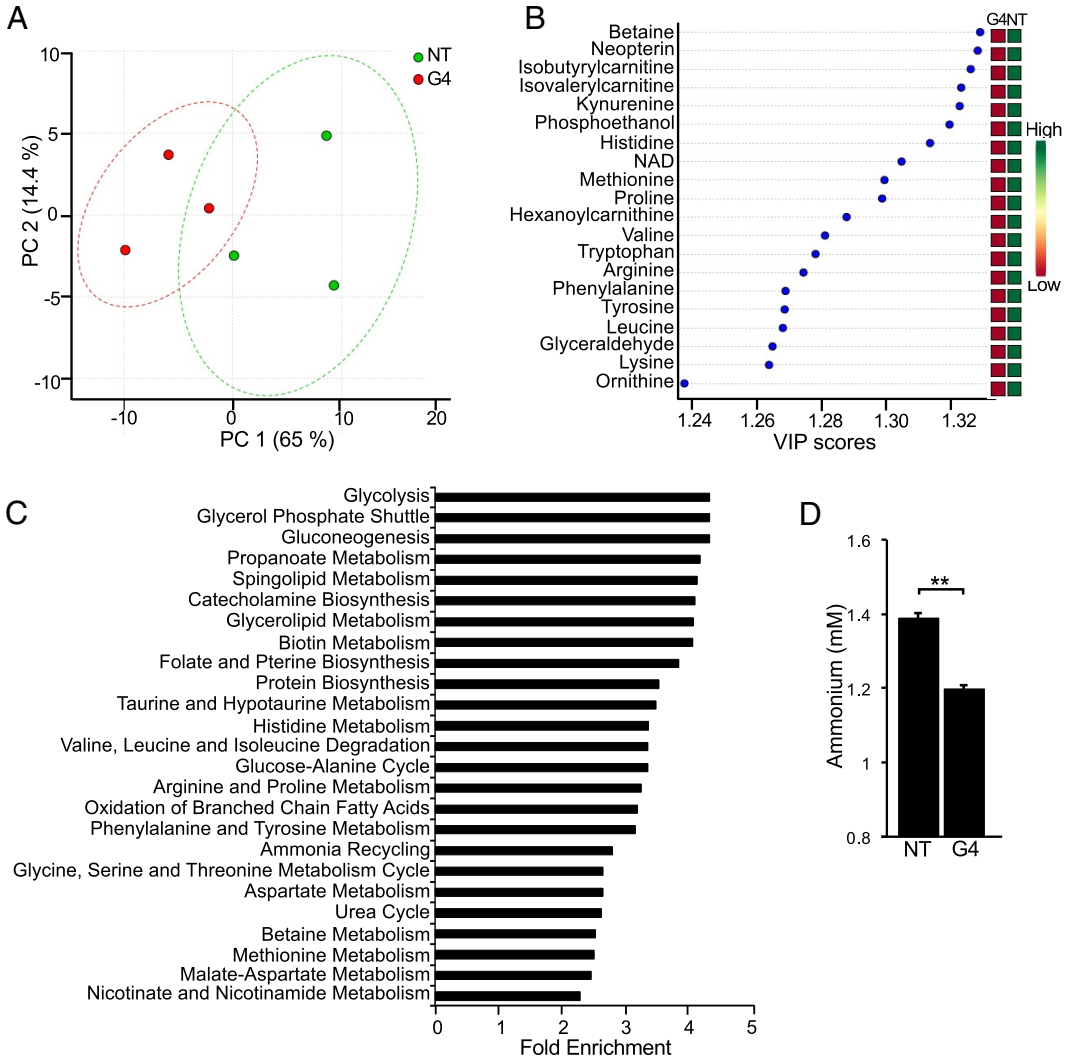

Figure 5. Altered metabolic profile in GATA4-depleted mLTC-1 cells. A total of 96 metabolites were analyzed for each sample group (n = 3) (A–C). A, Principle component analysis (PCA) revealed the unsupervised separation between of the 2 sample groups (*Gata4* siRNA and nontargeting siRNA treated) based on differences in their metabolic profiles. B, Partial least squares discriminant analysis (PLSDA) identified the 20 most significantly changed metabolites and their relative abundance in control and *Gata4*-manipulated cells. The components were sorted according to the variable of importance in the projection (VIP) for the first component. Relative magnitudes of each metabolite disturbance are listed on the right as high (green) and low (red). C, Quantitative enrichment analysis using a generalized linear model to estimate a Q-statistic for each metabolite set identified a total of 25 significantly changed ($P < .05$) metabolic pathways. D, Ammonium concentrations in conditioned media derived from mLTC-1 cells 72 hours after transfection was measured using Konelab Arena 20 XT. Values are expressed as the mean ± SD (**, $P < .01$; n = 8). NT, nontargeting siRNA; G4, *Gata4* siRNA.

development in 1 kindred, although the precise impact of this mutation on Leydig cell function is unclear (60). In the mouse, *Gata4*$^{-/-}$ chimera analysis and conditional mutagenesis experiments using *Gata4*$^{flox/flox}$ mice have yielded conflicting results. As highlighted in a recent review (2), the interpretation of conditional knockout studies in the mouse testis is fraught with difficulty owing to context-specific effects, compensatory mechanisms, and other factors.

Here, using reductionist approach with simplified experimental models, we provide genetic evidence that GATA4 is a key regulator of Leydig cell function. Silencing of *Gata4* in mLTC-1 cells and primary adult Leydig cells led to decreased expression of genes in the androgen biosynthetic pathway (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*, and *Srd5a*). In mLTC-1 cells this was accompanied by reduced production of sex steroid precursors (steroid levels in conditioned media from primary Leydig cell cultures were too low to be detected by mass spectrometry). Comprehensive metabolomic analysis of GATA4-deficient mLTC-1 cells showed disruption of other metabolic pathways, particularly glycolysis. Consistent with impaired glycolysis, GATA4-depleted mLTC-1 cells had reduced expression of glycolytic genes (*Hk1*, *Gpi1*, *Pfkp*, and *Pgam1*), lower intracellular levels of ATP, and increased extracellular lev-

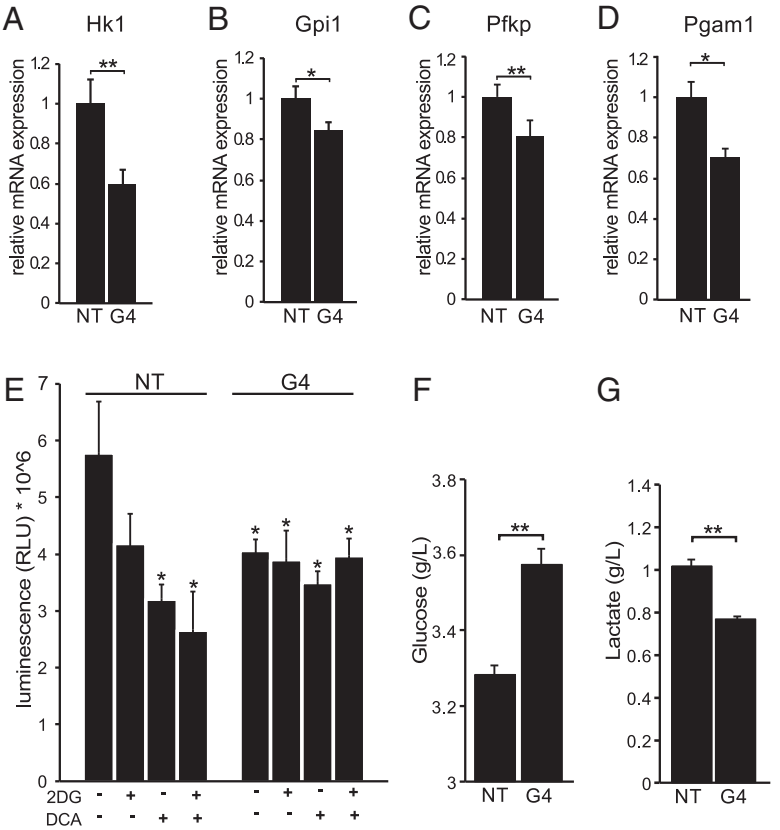

Figure 6. GATA4 depletion in mLTC-1 cells is associated with impaired glycolysis. Cells were treated with nontargeting (NT) siRNA or *Gata4* (G4) siRNA for 72 hours. A–D, The expression of glycolytic genes was analyzed using qRT-PCR. Results were normalized to *Actb* mRNA and are presented as relative expression values of the mean ± SD (**, $P < .01$; *, $P < .05$, n = 8). NT, NT siRNA; G4, *Gata4* siRNA. E, The contribution of the glycolytic pathway to ATP production was examined in *Gata4* siRNA-treated mLTC-1 cells and NT siRNA control cells. 2DG and DCA were used to inhibit glycolysis. Values are expressed as the mean ± SD. All bars were compared with NT siRNA control cells in the absence of inhibitors (*, $P < .05$; n = 4). F and G, Metabolite concentrations in conditioned media derived from mLTC-1 cells 72 hours after transfection was measured using Konelab Arena 20 XT. Values are expressed as the mean ± SD (**, $P < .01$; n = 8). NT, NT siRNA; G4, *Gata4* siRNA.

els of glucose. Our findings suggest that GATA4 plays a pivotal role in Leydig cell function and provide novel insights into metabolic regulation in this cell type.

In steroidogenic cells the binding of hormones to G-coupled receptors leads to activation of the cAMP/protein kinase A (PKA) pathway. PKA phosphorylates cAMP-responsive element binding protein (CREB), which activates transcription of a host of genes. Like CREB, GATA4 is phosphorylated in steroidogenic cells by PKA, and this enhances the intrinsic activity of GATA4 (61). Phosphorylated GATA4 has been shown to activate several cAMP-responsive genes, including those lacking a consensus binding site for CREB (eg, *Star*, *Cyp17a1*, *Cyp11a1*, and *Inha*) (61). These same genes were significantly downregulated in GATA4-depleted mLTC-1 cells and primary Leydig cells (see Figure 3), reinforcing the premise that GATA4 is a downstream effector of cAMP/PKA signaling.

In addition to regulating of steroidogenesis and glycolysis, GATA4 influences cell survival. We found that *Gata4* silencing is associated with a decrease in mLTC-1 cell viability, as measured by an MTS-based assay, and a concomitant increase in apoptosis. Of interest, we previously identified GATA4 as an antiapoptotic factor in human ovarian granulosa cells (62). In rodent cardiomyocytes, inhibition of *Gata4* leads to enhanced apoptosis mediated by members of B-cell lymphoma 2 protein family (63). However, no *Bcl2* related genes were found to be significantly dysregulated in our transcriptomic analysis of GATA4-deficient mLTC-1 cells. The increased apoptosis in GATA4-depleted mLTC-1 cells may reflect impaired ATP production, which has been shown to trigger apoptosis in other cancer cell lines (64). Disrupted autocrine signaling may also contribute to decreased cell viability in GATA4-depleted Leydig cells. We found that *Gata4* silencing of mLTC-1 cells and primary Leydig cells is associated with decreased expression of Kit, a known survival factor for mature Leydig cells and a growth factor for precursor Leydig cells (46).

Genome wide occupancy analyses have shown that GATA4 chromatin occupancy is tissue specific. Interestingly, a study by Zheng et al showed that GATA4 occupies promoter sites of genes associated with glycolysis in the context of hepatocytes but not cardiomyocytes (65). The notion that a single transcription factor can govern cellular growth/survival, steroidogenesis, glycolysis, and other metabolic pathways was shown recently in a study of nuclear receptor subfamily 5, group A, member 1 (AdBP4; SF1) in Y-1 mouse adrenocortical and MA-10 Leydig tumor cell lines (66). The authors found that NR5A1 regulates genes required to generate ATP and NADPH, 2 essential energy carriers used for the de novo synthesis of steroid hormones. Of note, all natural steroids in animal cells are derived from lanosterol, which is synthesized via cyclization of the triterpenoid squalene (67). It is of interest that the terpenoid biosynthetic pathway appeared to be one of

the most significant changed pathways in our transcriptomic KEGG pathway analysis.

During glycolysis, glucose is catabolized to generate ATP, essential for cellular function, biomass supply, and proliferation. Both transcriptomic and metabolomic analyses suggest that glycolysis is disrupted in GATA4-depleted Leydig tumor cells. Cytosolic NAD functions as a cofactor for key glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase and pyruvate dehydrogenase. The balance between the oxidized and reduced forms of NAD (NAD^+ to NADH ratio) is described as the redox state of a cell, a measure that reflects both the metabolic activity and general health of a cell (68). The NAD^+ to NADH ratio impacts not only cytosolic glycolysis but also mitochondrial oxidative phosphorylation require for a sufficient supply of NAD. After cytosolic NAD depletion, seen in *Gata4* compromised mLTC-1 cells, glucose can no longer be converted to pyruvate, which is needed to fuel mitochondrial oxidative phosphorylation.

Altered energy metabolism is a hallmark of tumor cells; the so-called Warburg effect describes the increased glycolytic capacity of tumor cells in the presence of oxygen (69). Consequently, caution must be exercised in extrapolating metabolic findings in tumor cells to primary tissue. Studies have shown that MA-10 Leydig tumor cells generate a significant proportion of their ATP from glycolysis, whereas primary Leydig cells appear to be almost completely dependent on mitochondrial respiration as their energy source (70). Thus, the changes in glycolysis and ATP production we observed in *Gata4*-depleted mLTC-1 Leydig tumor cells may or may not be applicable to primary Leydig cells, and future studies will test this. Recently, conditional mutagenesis of *Gata6* was shown to alter the expression of metabolic regulators critical for generation of acetyl coenzyme A in murine peritoneal macrophages, proving that GATA factor inhibition can impact intermediary metabolism in nontransformed cells (71).

While this manuscript was under revision, Bergeron et al reported the results of *Gata4* knockdown studies in mouse MA-10 Leydig tumor cells (72). As in mLTC-1 cells, inhibition of *Gata4* expression in MA-10 cells was associated with reduced expression of genes involved in the synthesis of androgens (eg, *Star*, *Cyp11a1*, *Hsd3b1*, and *Std5a*) and peptide hormones (eg, *Inha* and *Inhbb*). These findings, together with earlier reports, support the notion that GATA4 is a key transcriptional regulator of Leydig cell function.

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Review

GATA factors in endocrine neoplasia

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ABSTRACT

GATA transcription factors are structurally-related zinc finger proteins that recognize the consensus DNA sequence WGATAA (the GATA motif), an essential *cis*-acting element in the promoters and enhancers of many genes. These transcription factors regulate cell fate specification and differentiation in a wide array of tissues. As demonstrated by genetic analyses of mice and humans, GATA factors play pivotal roles in the development, homeostasis, and function of several endocrine organs including the adrenal cortex, ovary, pancreas, parathyroid, pituitary, and testis. Additionally, GATA factors have been shown to be mutated, overexpressed, or underexpressed in a variety of endocrine tumors (*e.g.*, adrenocortical neoplasms, parathyroid tumors, pituitary adenomas, and sex cord stromal tumors). Emerging evidence suggests that GATA factors play a direct role in the initiation, proliferation, or propagation of certain endocrine tumors via modulation of key developmental signaling pathways implicated in oncogenesis, such as the WNT/β-catenin and TGFβ pathways. Altered expression or function of GATA factors can also affect the metabolism, ploidy, and invasiveness of tumor cells. This article provides an overview of the role of GATA factors in endocrine neoplasms. Relevant animal models are highlighted.

Contents

MCE Special Edition: Animal Models of Endocrine Neoplasia

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1. Overview of GATA factors in development and disease

1.1. Members of the vertebrate GATA family

By regulating cell fate specification and differentiation, GATA transcription factors play important roles in eukaryotic development. The vertebrate GATA family comprises six members (GATA1–6) named in order of discovery (Patient and McGhee, 2002). All bind to the consensus DNA element, WGATAA, known as the GATA motif (Bresnick et al., 2012). With the exception of *Gata5*, homozygous null mutations in genes encoding GATA family members result in embryonic lethality in mice, underscoring the vital roles that these transcription factors play in development (reviewed in Molkentin, 2000 and Viger et al., 2008). Human diseases associated with germline loss-of-function mutations in GATA factors are summarized in Table 1.

All vertebrate GATA proteins contain a pair of conserved zinc finger domains (Patient and McGhee, 2002). The C-terminal finger is essential for DNA binding, whereas the N-terminal finger physically interacts with other transcriptional regulators such as the "Friend of GATA" factors FOG1 (ZFPM1) and FOG2 (ZFPM2) (Bresnick et al., 2012). GATA motifs are common in the genomes of vertebrates, but global chromatin immunoprecipitation studies in various cell types suggest that GATA factors occupy less than 1% of the consensus sites (Bresnick et al., 2012). This implies the existence of mechanisms that discriminate among these abundant motifs.

GATA factors can act as either transcriptional activators or repressors depending on the context (Viger et al., 2008). Although all GATA factors bind to the same DNA element, there is surprisingly little functional redundancy among these proteins (Zheng and Blobel, 2010). Individual GATA factors elicit distinctive functions through cooperative interactions with other transcriptional regulators. Functional diversity is also achieved via post-translational modifications (*e.g.*, phosphorylation, acetylation, and sumoylation) that alter the intrinsic activity of different GATA factors (Viger et al., 2008).

During the differentiation of a particular lineage, a GATA motif in the promoter/enhancer of a gene may be occupied sequentially by more than one GATA factor. For example, as erythroid progenitors mature the stem cell factor GATA2 is displaced from specific sites by the terminal differentiation factor GATA1, a phenomenon termed a "GATA switch" (Bresnick et al., 2010). A similar switch is presumed to occur in intestinal epithelium, where GATA6 maintains stem/progenitor cells and GATA4 promotes terminal differentiation into mature enterocytes (Beuling et al., 2011). GATA switches are often

Table 1
Human diseases associated with germline loss-of-function mutations in GATA factors.

Gene	Human disease phenotype	References
GATA1	• X-linked dyserythropoietic anemia and thrombocytopenia • X-linked Diamond–Blackfan anemia	Crispino and Weiss (2014)
GATA2	• Monocytopenia with mycobacterial infections (MonoMAC) • Dendritic cell, B, and natural killer (NK) lymphoid deficiency • Familial MDS/AML • Emberger syndrome (primary lymphedema with MDS)	Bresnick et al. (2012); Spinner et al. (2014)
GATA3	• HDR syndrome (hypoparathyroidism, deafness, and renal dysplasia)	Ali et al. (2007); Nesbit et al. (2004); Van Esch et al. (2000)
GATA4	• Congenital heart disease • Congenital diaphragmatic hernia • Monogenic diabetes • Aberrant testicular development	Lourenco et al. (2011); Prendiville et al. (2014); Shaw-Smith et al. (2014); Yu et al. (2013)
GATA5	• Congenital heart disease	Shi et al. (2014)
GATA6	• Congenital heart disease • Pancreatic agenesis and/or monogenic diabetes • Biliary tract abnormalities • Congenital diaphragmatic hernia	Allen et al. (2012); Bonnefond et al. (2012); Maitra et al. (2010); Prendiville et al. (2014); Yu et al. (2014)

associated with altered transcriptional output, emphasizing that different GATA factors can exert distinct functions at the same binding site (Bresnick et al., 2012). GATA switches occur not only during normal development but also in disease states, such as endometriosis. The transformation of endometrial stroma into endometriotic stroma is accompanied by an epigenetically-mediated switch from expression of *GATA2* to *GATA6* (Dyson et al., 2014).

1.2. GATA factors as tumor markers

Since GATA factors regulate genetic networks that can expand stem/progenitor cell populations or drive differentiation, it comes as no surprise that alterations in the expression or function of GATA factors have been linked to neoplastic transformation. Indeed, GATA factor genes have been shown to be mutated, overexpressed, or underexpressed in a wide range of solid tumors and leukemias (reviewed in Ayanbule et al., 2011; Bresnick et al., 2012; Viger et al., 2008; and Zheng and Blobel, 2010).

GATA factors can serve as tumor markers that shed light on the developmental origins, clinical behavior, and pathogenesis of certain neoplasms, as illustrated by studies of GATA3 in breast cancer and GATA2 in prostate cancer. GATA3 is abundantly expressed in luminal cells of the mammary epithelium but not their multipotential progenitors (Chou et al., 2010). Conditional deletion studies in the mouse have shown that *Gata3* is required for branching morphogenesis and terminal differentiation of luminal epithelial cells (reviewed in Chou et al., 2010 and Zheng and Blobel, 2010). Interestingly, loss of *Gata3* in adult mammary epithelium triggers de-differentiation of luminal cells, increased cell proliferation, and disorganization of ducts, features reminiscent of neoplastic transformation. In primary breast tumors low or absent *GATA3* expression is associated with shorter patient survival and a host of negative prognostic indicators (primary tumor size, lymph node metastases, lack of estrogen receptor and progesterone receptor expression, *etc.*) (Chou et al., 2010). GATA2 is expressed in

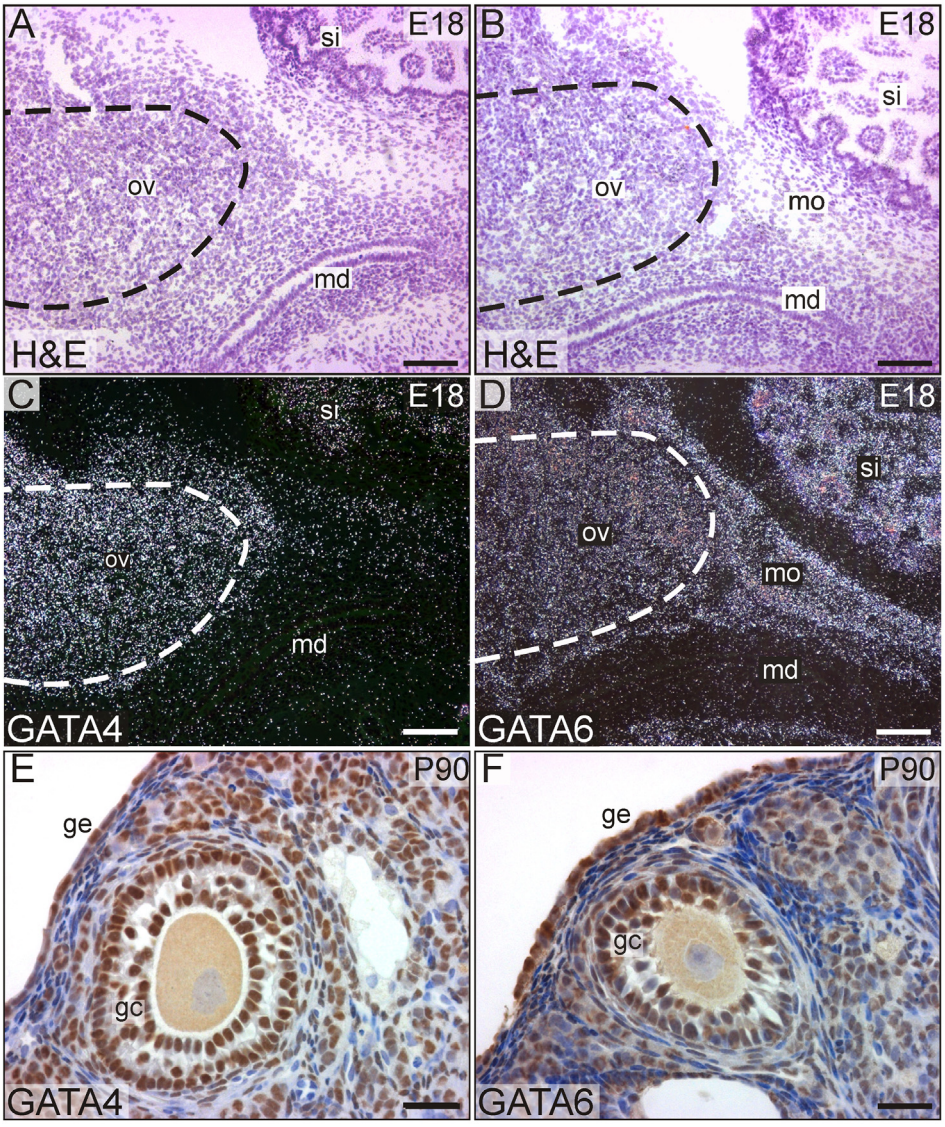

Fig. 1. Expression of *Gata4* and *Gata6* in developing mouse ovary. (A–D) Cryosections of E18.5 mouse were subjected to *in situ* hybridization for GATA4 or GATA6 using [^{33}P]-labeled riboprobes and counterstained with hematoxylin and eosin (H&E). Shown are corresponding brightfield (A,B) and darkfield (C,D) photomicrographs. GATA4 and GATA6 mRNA are evident in fetal ovary (ov) and small intestine (si). Additionally, GATA6 mRNA is seen in the mesovarium (mo) but not in the Müllerian duct (md). Bars = 100 μm. (E,F) Immunohistochemical staining of ovary from a parous female (P90) mouse. GATA4 and GATA6 proteins are evident in granulosa cells (gc) and germinal epithelium (ge). Bars = 50 μm.

both benign prostatic epithelium and prostate cancer, but levels of GATA2 are significantly higher in the latter and correlate with the risk of cancer progression and metastasis (He et al., 2014). Androgen receptor (AR) signaling is a key driver of prostate cancer, and GATA2 has emerged as a critical regulator of AR expression and activity in this malignancy (Chiang et al., 2014; He et al., 2014; Wu et al., 2014).

1.3. GATA factors interface with signaling pathways involved in both normal development and tumorigenesis

Signaling pathways that control stem cell self-renewal, terminal differentiation, and cell survival, such as the WNT/β-catenin and TGFβ pathways, are often co-opted during tumorigenesis. GATA factors have been shown to interface with developmental signaling pathways implicated in oncogenesis, as evidenced by studies of GATA6 in colorectal tumors. Constitutive activation of WNT/β-catenin signaling and inhibition of bone morphogenetic protein (BMP) signaling are the principal genetic alterations associated with colorectal tumor formation (Whissell et al., 2014). GATA6 plays a key role in colorectal tumorigenesis by driving expression of *LGR5*, which enhances WNT/β-catenin signaling and enables tumor stem cell renewal, and by inhibiting expression of BMPs, which promote terminal differentiation (Tsuji et al., 2014; Whissell et al., 2014). GATA factors have also been shown to regulate genes involved in apoptosis of normal and tumor cells. For example, GATA4 has been shown to protect cardiomyocytes from doxorubicin-induced apoptosis by upregulating anti-apoptotic members of the BCL2 protein family (Aries et al., 2004; Kobayashi et al., 2010) and to protect ovarian tumor cells from an extrinsic apoptosis inducing ligand TRAIL (Kyrönlahti et al., 2010) (see Section 2.2).

1.4. GATA factor dysregulation can affect tumor cell migration, invasiveness, and ploidy

GATA factor dysregulation can impact tumor cell biology in other ways. In breast cancer cells GATA3 promotes differentiation, limits cell migration, and suppresses metastasis by inducing expression of a microRNA (miR-29b) that downregulates expression of genes involved in angiogenesis, collagen remodeling, and proteolysis (Chou et al., 2013). Ovarian carcinomas are thought to arise from ovarian surface (germinal) epithelium or neighboring oviduct epithelium (Nezhat et al., 2015). Approximately 50% of ovarian carcinomas lack *GATA6* expression entirely, and an additional 40% show abnormal GATA6 immunostaining that is either weak or cytoplasmic rather than nuclear (Cai et al., 2009). Loss of GATA6 in germinal epithelial cells triggers their de-differentiation, manifested as the loss of expression of proteins required for epithelial organization (Cai et al., 2009; Capo-chichi et al., 2009, 2011). Loss of *GATA6* expression in these cells also leads to deformation of the nuclear envelop and a failure of cytokinesis, resulting in aneuploidy (Capo-chichi et al., 2009). The link between GATA6 deficiency and aneuploidy appears to be a generalized phenomenon, because mouse peritoneal macrophages lacking *Gata6* expression also exhibit changes in ploidy along with metabolic derangements (Gautier et al., 2014; Rosas et al., 2014).

1.5. Focus of this article

This article reviews the role of GATA factors in neoplasias of various endocrine tissues. The expression patterns of GATA factors in developing endocrine organs and in their corresponding neoplasms are described. The use of GATA factors as endocrine tumor markers in both preclinical and clinical settings is discussed. Relevant animal models, such as the mouse, ferret, dog, and goat, are highlighted. Endocrine-related tumors, such as breast and prostate cancer, are not a focus of this review.

Dozens of putative GATA target genes have been identified in endocrine tissues. Unfortunately, space constraints do not allow us to cite all the original research papers characterizing these target genes. Instead, the reader is referred to review articles that summarize GATA target genes in endocrine tissues (LaVoie, 2003; Röhrig et al., 2015; Tevosian, 2014; Viger et al., 2008).

2. Ovarian neoplasms

2.1. Roles of GATA4 and GATA6 in ovarian development and function

GATA4 and GATA6 are the predominant GATA factors expressed in the developing ovary (Fig. 1) (LaVoie, 2014; Viger et al., 2008). At embryonic day (E) 10.5 in the mouse, *Gata4* expression is evident in the genital ridge (Hu et al., 2013), and by E13.5 GATA4 is found in most ovarian somatic cells (Anttonen et al., 2003; Efimenko et al., 2013; Heikinheimo et al., 1997; Kyrönlahti et al., 2011b). In the adult ovary, GATA4 is present in theca cells and in granulosa cells of primary, preantral, and antral follicles, but not in primordial follicles or luteal cells (LaVoie et al., 2004; Viger et al., 2008). Like *Gata4*, *Gata6* is expressed in somatic cells of the prenatal ovary and in theca and granulosa cells of large follicles in the adult ovary; however, in contrast to *Gata4*, *Gata6* is expressed in corpora lutea (Heikinheimo et al., 1997; Miyamoto et al., 2008). Both GATA4 and GATA6 are found in ovarian surface epithelium (Capo-chichi et al., 2003).

Ovarian expression of GATA factors is controlled by an array of endocrine and paracrine factors, including gonadotropins and members of the TGFβ superfamily (reviewed in Viger et al., 2008). For example, treatment of juvenile mice with eCG enhances follicular expression of *Gata4* and *Gata6* (Heikinheimo et al., 1997), while treatment of cultured preovulatory human granulosa cells with hCG upregulates GATA6 expression (Laitinen et al., 2000). In granulosa cell tumors, TGFβ treatment increases GATA4 levels (Anttonen et al., 2006). In ovarian somatic cells and other endocrine tissues, GATA factors serve to integrate input from signaling pathways including the cyclic AMP/protein kinase A (cAMP/PKA) and mitogen-activated protein kinase (MAPK) pathways (reviewed in Viger et al., 2008). Activation of these two signaling pathways in gonadal cells results in phosphorylation of GATA4 at distinctive sites that mediate synergistic interactions with other transcriptional regulators (reviewed in Viger et al., 2008). Promoter analyses have identified a number of GATA-dependent genes in ovarian steroidogenic cells, including *Star*, *Cyp11a1*, *Cyp19a1*, *Hsd17b1*, and *Inha* (reviewed in Viger et al., 2008). Gene targeting studies in the mouse have delineated the importance of *Gata4* and *Gata6* in follicular development and ovarian function (Table 2) (reviewed in Tevosian, 2014).

2.2. Granulosa cell tumors

Granulosa cell tumors (GCTs), the most common sex-cord stromal tumors, are subclassified in two forms: an adult-type (AGCT), that typically occurs in perimenopausal women, and a rare juvenile form that affects mostly children and adolescents (Schumer and Cannistra, 2003). GCTs are steroidogenically active and can cause precocious puberty, disturbances in the menstrual cycle, and endometrial hyperplasia (Jamieson and Fuller, 2012; Schumer and Cannistra, 2003).

AGCTs retain the biological features of normal proliferating granulosa cells of preovulatory follicles (Fuller et al., 2002). Thus, the molecular pathogenesis of AGCT is hypothesized to entail

Table 2
Ovarian phenotypes of *Gata4* and *Gata6* conditional knockout mice.

Gene(s)	Cre	Phenotype	References
Gata4	*Sf1*-cre	Impaired granulosa/theca cell proliferation, depletion of primordial follicles, ovarian cyst formation, and sterility	Efimenko et al. (2013)
	Amhr2-cre	Cystic ovarian changes, impaired fertility, and a blunted response to exogenous gonadotropins	Kyrönlahti et al. (2011b)
Gata6	*Sf1*-cre	No overt phenotype	Pihlajoki et al. (2013)
	Amhr2-cre	Early embryonic lethal owing to leaky expression in yolk sac endoderm	Wilson (unpublished observations)
Gata4 & *Gata6*	*Cyp19*-cre	Impaired formation of large pre-ovulatory follicles and corpora lutea, an abnormal estrous cycle, and infertility; impaired expression of *Fshr*, *Lhcgr*, *Inha*, and *Inhbb*	Bennett et al. (2012, 2013)
	Sf1-cre	Aberrant folliculogenesis and impaired expression of genes involved in early ovarian development, including *Foxl2* and *Fst*	Padua et al. (2014)

disrupted expression of signaling pathways that regulate granulosa cell proliferation and apoptosis. A somatic missense mutation (p.C134W) in *FOXL2*, a transcription factor required for normal murine granulosa cell differentiation and ovarian maintenance (Schmidt et al., 2004; Uhlenhaut et al., 2009), is present in ~95% of AGCTs, suggesting that it is pathognomonic for this tumor (Jamieson et al., 2010; Kim et al., 2010b; Shah et al., 2009). In contrast, juvenile GCTs lack the p.C134W mutation (Kalfa et al., 2007).

Current evidence suggests that a key event in AGCT pathogenesis is a failure of the mutant FOXL2 to form specific protein–protein interactions, leading to subtle changes in the transcription of target genes (L'Hote et al., 2012). Recent studies suggest that GATA4 cooperates with FOXL2 during granulosa cell tumorigenesis (Fig. 2). The majority of AGCTs express *GATA4* at levels comparable to normal preovulatory granulosa cells (Laitinen et al., 2000), and high GATA4 expression in these tumors predicts both increased risk of recurrence and shorter disease specific survival (Färkkilä et al., 2014). GATA4 expression in AGCTs also correlates with the intrinsic apoptotic pathway inhibitor BCL2 and proproliferative CCND2 expression, suggesting that GATA4 may act as an anti-apoptotic factor in adult AGCTs (Kyrönlahti et al., 2008). GATA4, SMAD3, and FOXL2 physically interact and modulate gene expression, cell viability, and apoptosis in AGCTs (Anttonen et al., 2014). GATA4 is also expressed in juvenile GCTs (Virgone et al., 2012); however, increased expression of GATA4 does not correlate with aggressive behavior as seen in adults. In contrast to GATA4, GATA6 expression in AGCTs is inversely correlated with tumor size, suggesting that GATA6 may suppress proliferation in this cell type (Anttonen et al., 2005).

A number of transgenic mouse models have been generated to examine the pathogenesis of AGCTs (Table 3). Aberrant expression of GATA factors accompanies tumorigenesis in several of these mouse models. Collectively, these models reinforce the importance of SMADs and GATA factors in the genesis of AGCTs.

2.3. Sertoli–Leydig cell tumors of the ovary

Sertoli–Leydig cell tumors (STLCs) are rare ovarian sex cord-stromal tumors characterized by proliferation of Sertoli and Leydig cells of varying degrees of differentiation (Zhang et al., 2014). Sertoli cells, not Leydig cells, are thought to constitute the neoplastic component of these tumors (Nouriani et al., 2002). The majority of SLCTs are diagnosed in adolescents or young adults. One-third of patients exhibit hyperandrogenic manifestations (acne, male-pattern baldness, *etc.*) (Zanotti, 2002; Zhang et al., 2014). A small percentage of SLCTs have estrogenic manifestations, such as menometrorrhagia or postmenopausal bleeding.

SLCTs are one of the characteristic tumors in the pleuropulmonary blastoma familial tumor predisposition syndrome,

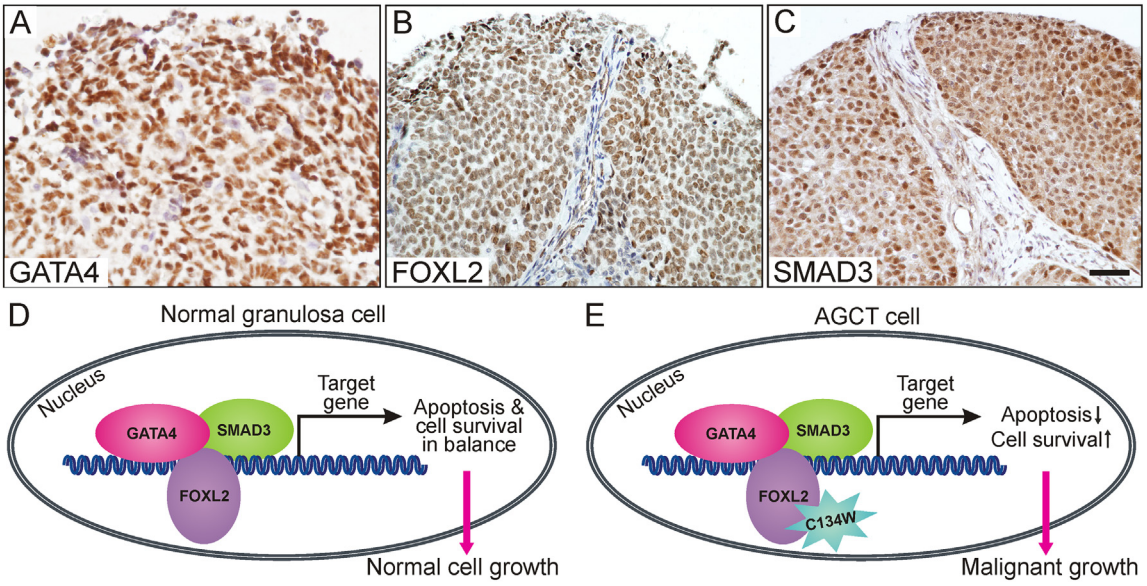

Fig. 2. Interplay among GATA4, FOXL2, and SMAD3 during human granulosa cell tumor development. Sections of formalin-fixed AGCT were subjected to immunohistochemical staining for (A) GATA4, (B) FOXL2, and (C) SMAD3. Bar = 50 μm. Normal granulosa cell growth is modulated by cooperative interactions among wild-type FOXL2, GATA4, and SMAD3 (D). The C134W mutation in *FOXL2* disrupts this balance leading to malignant cell growth (E).

Table 3
Genetically-engineered mouse models of GCT.

Genotype	Phenotype	Tumor penetrance (%)	References
Inha-TAg	Infertile, GCT formation at age of 5–7 mo, serum inhibin B ↑, serum gonadotropins ↓	100	Kananen et al. (1995)
Inha$^{-/-}$	Bilateral, mixed, or incompletely differentiated sex cord-stromal tumors coupled with cachexia-like syndrome at age of 4 wks	100	Matzuk et al. (1992)
Smad1/5 double knockout	Infertile, poorly differentiated, metastatic, uni- or bilateral GCT at age of 3 mo, histopathological features similar to human juvenile GCT	100	Pangas et al. (2008); Middlebrook et al. (2009)
Smad1/5/8 triple knockout	Infertile, poorly differentiated, metastatic, uni- or bilateral GCT at age of 3 mo	100	Pangas et al. (2008)
Catnb$^{flox(ex3)/+}$; *Amhr2*$^{cre/+}$	Subfertile, follicle-like lesions that evolve into GCT at 7.5 mo of age	57	Boerboom et al. (2005)
Men1$^{+/-}$	Develop GCTs, a feature not typical of humans with the MEN1 cancer predisposition syndrome; *Gata6* is downregulated in GCTs that arise in the ovaries of these mice	70	Mould et al. (2009)

which is caused by heterozygous germline mutations in the microRNA maturation gene *DICER1* (Schultz et al., 2014). More than half of SLCTs harbor mutations in *DICER1* (Witkowski et al., 2013). Conditional deletion experiments in mice have established that *Dicer1* regulates gonadal somatic cell function (Huang and Yao, 2010; Kim et al., 2010a; Lei et al., 2010; Nagaraja et al., 2008), but sex cord stromal tumors have not been reported in germline or conditional *Dicer1* knockout mice.

Based on its established role in testicular somatic cell differentiation (see Section 3.1), GATA4 is hypothesized to have a role in pathogenesis of ovarian SLCTs. A series of studies have shown that GATA4 and its cofactor FOG2 are expressed in some but not all SLCTs (Ketola et al., 2000; Mosbech et al., 2014; Siltanen et al., 1999; Virgone et al., 2012). Currently, however, there is no genetic or epigenetic data linking altered expression or function of GATA factors to SLCTs.

2.4. Thecoma–fibroma tumors of the ovary

Thecoma–fibroma is a group of benign ovarian sex cord-stromal tumors. These rare neoplasms are composed of varying amounts of theca cells and fibroblasts (Chen et al., 2003). This group of tumors is subdivided into three categories: (1) thecoma, containing lipid-laden theca cells without fibroblasts, (2) thecoma–fibroma, containing both theca cells and fibroblasts, and (3) fibroma, composed almost entirely of fibroblasts. These tumors can occur at any age, but menopausal and postmenopausal women account for most of the cases (Chen et al., 2003). Patients typically present with an abdominal mass and attendant pain; functional tumors may be associated with irregular menstrual bleeding.

Young women with Gorlin (nevoid basal cell carcinoma) syndrome, a tumor predisposition disorder associated with excessive hedgehog signaling due to heterozygous mutations in *PTCH1*, develop bilateral ovarian fibromas (Morse et al., 2011). One boy with Gorlin syndrome and a testicular thecoma–fibroma has been reported (Ueda et al., 2010). Loss of heterozygosity at 9q22.3, which harbors the *PTCH1* gene, is observed in 40% of sporadic ovarian thecoma–fibroma cases (Tsuji et al., 2005), supporting a pathogenic association between aberrant hedgehog signaling and thecoma–fibroma development. Abundant expression of GATA4 and its cofactor FOG2 have been reported in two pediatric cases of thecoma–fibroma, including a case of bilateral ovarian fibroma associated with Gorlin syndrome (Virgone et al., 2012).

3. Testicular neoplasms

3.1. GATA factors implicated in testicular development and function

During fetal testicular development *Gata4* is expressed in Sertoli cells, fetal Leydig cells, and peritubular myoid cells (Table 4) (Bielinska et al., 2007; Mazaud-Guittot et al., 2014). In the adult testis *Gata4* is expressed in Sertoli cells, Leydig cells, and putative stem Leydig cells (Ketola et al., 1999, 2002; Kilcoyne et al., 2014; Landreh et al., 2014; McCoard et al., 2001; Oreal et al., 2002). Like GATA4, GATA6 is found in testicular somatic cells. In the mouse, testicular *Gata6* expression begins in Sertoli cells at E14.5 (Robert et al., 2002) and in fetal Leydig cells shortly thereafter (Tevosian, 2014). Postnatally, Sertoli cells continue to express *Gata6* through to adulthood (Anttonen et al., 2003; Imai et al., 2004; Ketola et al., 1999, 2002, 2003; Oreal et al., 2002). *Gata1* is also expressed in mouse Sertoli cells, albeit in a stage-specific manner (stages VI–IX of the seminiferous epithelial cycle) that is dependent on the presence of maturing germ cells (Ketola et al., 2002; Yomogida et al., 1994). Both GATA4 and GATA6 have been detected in adult

Table 4
Testicular phenotypes of *Gata4* and *Gata6* conditional knockout mice.

Gene(s)	Cre	Phenotype	References
Gata4	*Sf1*-cre	Decreased expression of *Dmrt1*; germ cell attrition	Manuylov et al. (2011)
	Amhr2-cre	Late onset impairment of Sertoli cell function with loss of fertility	Kyrönlahti et al. (2011b)
	Wt1-creERT2	Conditional ablation at E10.5 leads to a block in testicular differentiation and male-to-female sex reversal	Manuylov et al. (2011)
	CAG-creER	Impaired formation of the genital ridge and subsequent licensing of primordial germ cells	Hu et al. (2013, 2015)
Gata6	*Sf1*-cre	No overt phenotype	Pihlajoki et al. (2013)
	Amhr2-cre	Early embryonic lethal owing to leaky expression in yolk sac endoderm	Wilson (unpublished observations)
Gata4 & *Gata6*	*Sf1*-cre	Impaired spermatogenesis, absence of Leydig cells, and ectopic appearance of adrenocortical-like cells	Padua et al. (2015)

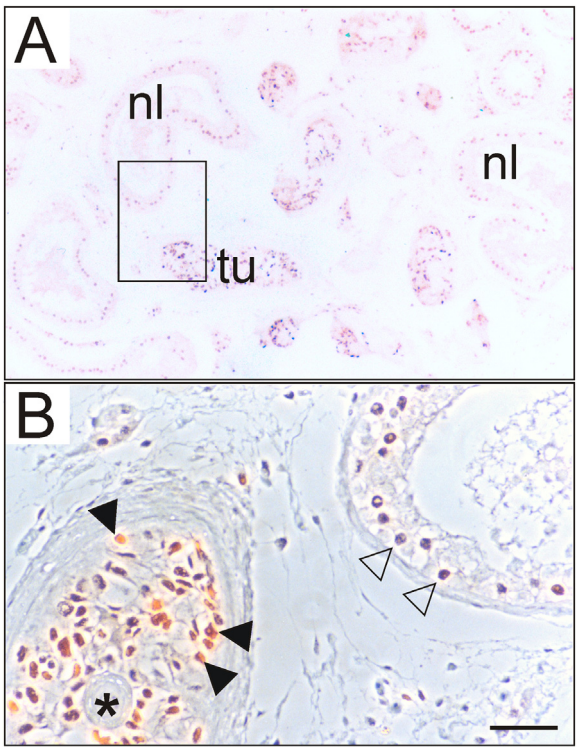

Fig. 3. Immunohistochemical staining of GATA4 in a human large cell calcifying Sertoli cell tumor. The box in panel A is shown at higher magnification in panel B. The asterisk highlights a calcified granule in the Sertoli cell tumor. Note that GATA4 is abundantly expressed in Sertoli (arrowheads) tumor cells. The adjacent normal Sertoli cells (open arrowheads) also express GATA4. Abbreviations: nl, normal seminiferous tubule; tu; tumor. Bar = 50 μm.

Leydig cells (Bielinska et al., 2007; Ketola et al., 1999, 2003), although GATA4 is the predominant GATA factor in this steroidogenic cell type.

Promoter analyses and related studies have identified several groups of putative target genes for GATA4 in testis, including genes associated with sex determination (*Sry*, *Sox9*, *Dmrt1*), peptide hormone production (*Inha*, *Inhb*, *Amh*), gonadotropin signaling (*Fshr*, *Lhcgr*), steroid synthesis (*Star*, *Cyp11a1*, *Cyp17a1*), and cell–cell interactions (*Clmp*, *Cldn11*, *Cx30.2*) (reviewed in Tevosian, 2014; Viger et al., 2008). In both Sertoli and Leydig cells, GATA4 activity is modulated via cooperative interactions with other transcriptional regulators/cofactors including SF1, liver receptor homolog 1 (LRH-1/NR5A2), FOG1, and FOG2 (reviewed in Viger et al., 2008). FOG proteins do not bind directly to DNA, but they can function as either enhancers or repressors of GATA transcriptional activity depending on the cell and promoter context studied; on gonadal promoters, however, FOG proteins appear to play a strictly repressive role (Tevosian, 2014). As in ovarian cells, GATA4 is a target for post-translational modifications such as phosphorylation (Tremblay and Viger, 2003; Viger et al., 2008).

Analysis of genetically-engineered mice has shown that interactions between GATA4 and its cofactor, FOG2, regulate the differentiation and function of fetal and adult Sertoli cells (reviewed in Tevosian, 2014). *Fog2*$^{-/-}$ mice and *Gata4*$^{ki/ki}$ mice, which bear a knock-in mutation that abrogates the interaction of GATA4 with FOG cofactors, exhibit similar testicular phenotypes including decreased testicular *Sry* expression, aberrant differentiation of Sertoli cells, and sex reversal (Bouma et al., 2007; Manuylov et al., 2007; Tevosian et al., 2002). More recently, conditional mutagenesis studies have shown that functional GATA4 is required for genital ridge development, testis cord morphogenesis, and Sertoli cell function (Hu et al., 2013, 2015; Kyrönlahti et al., 2011a; Manuylov et al., 2011).

The role of GATA4 in Leydig cell development, however, has remained unclear, because gene targeting experiments in mice have not shown a consistent phenotype (reviewed in Tevosian, 2014). For example, *Gata4*$^{-/-}$ progenitors exhibit an impaired capacity to differentiate into fetal Leydig cells in the testis of chimeric mice (Bielinska et al., 2007). In contrast, conditional ablation of *Gata4* in Leydig cells as early as E12.5 does not cause an overt impairment in the expression of Leydig cell differentiation markers in the fetal or adult testis (Manuylov et al., 2011). Interpreting the results of gene targeting experiments in the mouse testis is challenging because of context-dependent effects, compensatory responses, alternative pathways of differentiation, and functional redundancy (Tevosian, 2014). To circumvent these limitations, the impact of *Gata4* deficiency on Leydig cell function has been analyzed in less complicated experimental models: two immortalized mouse Leydig tumor cell lines (MA-10, mLTC-1) and primary cultures of adult mouse Leydig cells (Bergeron et al., 2015; Schrade et al., 2015). Using siRNA and related knockdown approaches, *Gata4* deficiency has been shown to have profound effects on specific metabolic pathways, notably steroidogenesis and glycolysis.

A missense mutation in the human *GATA4* gene has been linked to abnormal testicular development in one kindred, although the precise impact of this mutation on somatic cell function is unclear (Lourenco et al., 2011). More recently, mutations in *FOG2* have been demonstrated in unrelated individuals with 46,XY gonadal dysgenesis (Bashamboo et al., 2014).

Despite the intriguing, stage-specific expression pattern of GATA1 in testis, gene targeting experiments in the mouse suggest that GATA1 is not essential for Sertoli cell function (Lindeboom et al., 2003).

3.2. GATA4 is expressed in large-cell calcifying Sertoli tumors

GATA4 expression has been reported in large-cell calcifying Sertoli tumors (LCCSCT), one of the sex cord tumors of testis (Fig. 3) (Ketola et al., 2000). LCCSCTs produce estrogen and are associated with gynecomastia and advanced skeletal maturation (Gourgari et al., 2012). Most cases of LCCSCT are sporadic, but about 40% are associated multiple neoplasia syndromes such as Peutz–Jeghers syndrome (PJS) or Carney complex (CNC). PJS is caused by loss-of-function mutations in the *STK11* gene, which inhibit AMP-activated protein kinase, resulting in increased activity of the mammalian target of rapamycin (mTOR) (Gourgari et al., 2012). Dysregulation of the mTOR pathway has been linked to tumorigenesis in various tissues, including endocrine tissues (de Joussineau et al., 2014). CNC is caused by *PRKAR1A* mutations, the gene encoding regulatory subunit type 1 of protein kinase A. This leads to excessive cAMP and mTOR signaling (de Joussineau et al., 2014; Sahut-Barnola et al., 2010). Whether signaling activation in these tumor disposition syndromes is associated with altered phosphorylation of GATA4 is unknown. Lending credence to this possibility, increased expression of GATA4 is evident in the adrenal glands of *Prkar1a* knockout mice signaling (Sahut-Barnola et al., 2010).

3.3. GATA4 is expressed in canine testicular tumors

A comprehensive survey of canine testicular tumors documented strong GATA4 immunoreactivity in all Sertoli cell tumors and the vast majority of (27/28) Leydig (interstitial) cell tumors (Ramos-Vara and Miller, 2009). Mixed germ cell sex cord-stromal tumors (MGSCT) in this species also expressed GATA4.

3.4. Gata6 is downregulated in an experimental model of Leydig cell adenoma

Heterozygous loss-of-function mutations in *Men1*, encoding a chromatin remodeling gene, predispose mice to the development of multiple endocrine tumors, recapitulating the human *MEN1* cancer predisposition syndrome. Additionally, *Men1*$^{+/-}$ mice develop gonadal somatic cell tumors, a feature not typical of humans with this cancer predisposition syndrome. Female *Men1*$^{+/-}$ mice develop GCTs that underexpress *Gata6* (Table 3), while their male counterparts develop Leydig cell tumors that underexpress *Gata6* (Mould et al., 2009). Loss of heterozygosity at the *Men1* locus is evident in these gonadal tumors, suggesting a direct link between *Men1* gene inactivation and tumorigenesis in this model (Bertolino et al., 2003; Hussein et al., 2007). Decreased expression of *Gata6* represents an attractive candidate for mediating gonadal somatic cells tumorigenesis in this model because: (1) GATA6 affects TGFβ signaling in other tumors such as colorectal neoplasms (see Section 1.4), (2) dysregulated TGFβ superfamily signaling accompanies Leydig cell tumorigenesis in the *Men1*$^{+/-}$ mice (Hussein et al., 2008), and (3) targeted mutagenesis of genes involved in TGFβ signaling (*e.g.*, *Inha*, *Amh*, *Amhr2*) have been linked to testicular and ovarian somatic cell tumors in mice (Behringer et al., 1994; Matzuk et al., 1995; Mishina et al., 1996) (Table 3).

3.5. Testicular adrenal rest tumors

Leydig cells in the adult testis can arise from different populations of stem/progenitor cells, including undifferentiated mesenchymal cells in the testicular interstitium, vascular progenitors, and peritubular cells (Davidoff et al., 2004; Landreh et al., 2014; Mendis-Handagama and Ariyaratne, 2001). Men with disrupted adrenocortical function due to CYP21 or CYP11B1 deficiency develop neoplastic nodules of hormonally-active adrenocortical tissue in the testis (testicular adrenal rest tumors, TARTs), thought to arise from one of these reservoirs of pluripotential stem/progenitor cells (Reisch et al., 2013; Val et al., 2006). TARTs express adrenocortical-specific genes (*CYP11B1*, *CYP11B2*, and *MC2R*) at much higher levels than adjacent testicular tissue (Smeets et al., 2015). In addition, TARTs express the Leydig cell markers such as *HSD17B3*. These findings reinforce the premise that TARTs may arise from a totipotent embryonic cell type in response to hormonal dysregulation. The endocrine and paracrine factors that drive TART growth are not fully understood. A longitudinal analysis of men with CYP21 deficiency found no association between the presence of TARTs and parameters of disease control with exogenous glucocorticoids ± mineralocorticoids (Reisch et al., 2013). *Gata4*/*Gata6* double knockout mice generated with *Sf1*-cre develop TART-like cells that produce glucocorticoids (Padua et al., 2015). This GATA-deficient mouse model may be useful for exploring the signals that drive TART formation in humans (Heikinheimo et al., 2015).

4. Adrenocortical neoplasms

4.1. GATA6 and GATA4 have been implicated in adrenocortical development and function

GATA6 is the principal GATA factor expressed in the adrenal cortex. *Gata6* is expressed diffusely in the adrenal cortex of the fetal mouse (Kiiveri et al., 2002). Postnatally, expression of *Gata6* in the mouse adrenal is limited to capsular and subcapsular cells (Pihlajoki et al., 2013). In primates, *GATA6* is expressed in the zona reticularis, where it is thought to regulate androgen biosynthesis (Jimenez et al., 2003; Nakamura et al., 2007, 2009). By comparison, GATA4 has a more restricted pattern of expression during adrenocortical development and is presumed to have a more limited role in the function of this organ (Kiiveri et al., 2002). During human development, GATA4 mRNA is evident in the fetal zone of the adrenal, but there is only weak expression of this transcript in the adrenal cortex postnatally. Similarly, *Gata4* is transiently expressed in the mouse adrenal cortex during fetal but not postnatal development.

Consistent with its proposed role in the biosynthesis of adrenocorticoids and androgens, GATA6 has been shown to enhance the transcription of *CYP11A1*, *CYP17A1*, *CYB5*, *SULT2A1*, and *HSD3B2* in cell lines (reviewed in Röhrig et al., 2015). GATA4 can substitute for GATA6 in *trans*-activation studies of the *CYP17A1* promoter (Flück and Miller, 2004), suggesting that GATA4 may serve to augment *CYP17A1* expression during fetal development.

The impact of GATA6 on adrenocortical development and physiology has been assessed through conditional gene deletion using *Sf1*-cre (Pihlajoki et al., 2013). *Gata6* conditional knockout mice exhibit a pleiotropic phenotype that includes: (1) a thin, cytomegalic adrenal cortex, (2) decreased expression of the zG differentiation marker *Cyp11b2*, (3) blunted production of glucocorticoids in response to exogenous ACTH, (4) the spontaneous accumulation of non-steroidogenic cells expressing gonadal-like markers, (5) ectopic chromaffin cells, and (6) the absence of an adrenal X-zone. Based on analogous conditional deletion studies of *Gata6* in pulmonary (Tian et al., 2011; Zhang et al., 2008) and intestinal epithelia (Beuling et al., 2011, 2012), GATA6 is hypothesized

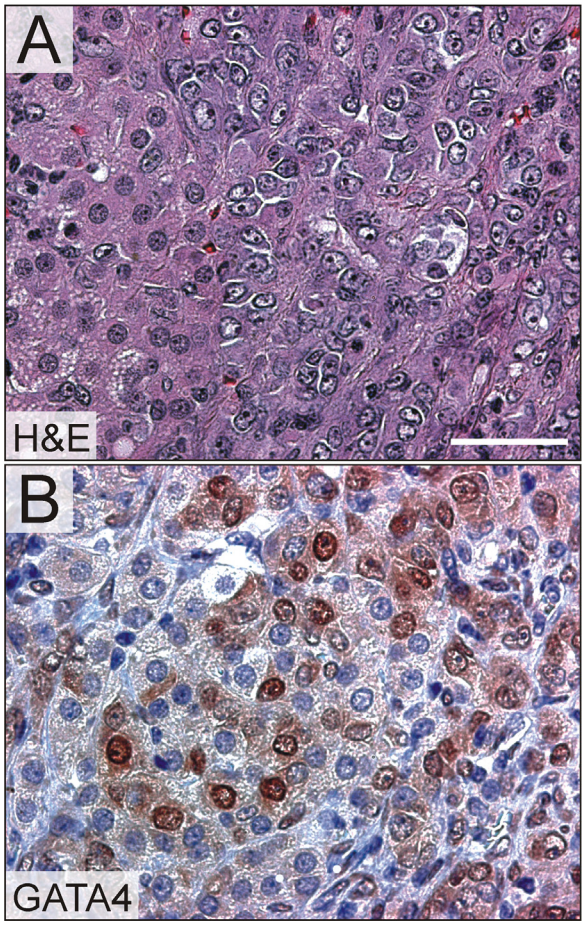

Fig. 4. Immunohistochemical staining of GATA4 in a GDX-induced adrenocortical carcinoma of the ferret. Serial sections of formalin-fixed tissue were stained with (A) hematoxylin and eosin (H&E), or (B) GATA4 antibody, followed by hematoxylin counterstaining. Bar = 50 μm.

to regulate the balance between stem/progenitor cell expansion and differentiation in the adrenal cortex. Targeted ablation of *Gata4* in SF1$^+$ cells has no significant impact on adrenocortical development, but *Gata4*/*Gata6* double mutant mice exhibit adrenocortical aplasia (Padua et al., 2015).

4.2. Gonadectomy-induced adrenocortical neoplasia

Gonadectomy (GDX) triggers the appearance of gonadal-like neoplasms in the adrenal cortex of mice (Röhrig et al., 2015). This phenomenon, termed GDX-induced adrenocortical neoplasia, is thought to reflect the metaplastic differentiation of stem cells in the adrenal capsule/subcapsule in response to the hormonal changes that accompany GDX (↑LH, ↓inhibin, *etc.*). The neoplastic cells express *Gata4*, *Lhcgr*, *Inha*, and enzymes required for sex steroid biosynthesis (*Cyp17a1*, *Hsd17b3*, *Cyp19a1*) (Bielinska et al., 2006; Schillebeeckx et al., 2015). Prototypical adrenocortical markers, such as *Gata6* and adrenocorticoid biosynthetic enzymes (*Cyp21a1*, *Cyp11b1*, *Cyp11b2*), are downregulated in the neoplastic adrenal tissue (Bielinska et al., 2006). In the mouse GDX-induced adrenocortical neoplasia is strain dependent, and chimera studies suggest that strain susceptibility to GDX-induced neoplasia is cell-intrinsic and resides in the stem/progenitor compartment (Röhrig et al., 2015). The genetic basis of strain susceptibility, however, remains unclear. Linkage analysis of crosses between susceptible (DBA/2J) and non-susceptible (C57Bl/6) mouse strains has shown that GDX-induced adrenocortical neoplasia is a complex trait (Bernichtein et al., 2007).

Loss- and gain-of-function studies have established that GATA4 directly modulates GDX-induced adrenocortical neoplasia. Constitutive and conditional mutations in *Gata4* mitigate the accumulation of gonadal-like neoplastic cells and the expression of sex steroidogenic markers in the adrenal cortex of gonadectomized female mice (Krachulec et al., 2012). Transgenic expression of *Gata4* in the adrenal cortex using a *Cyp21a1* promoter induces adrenocortical neoplasia in a non-susceptible strain (C57Bl/6) (Chrusciel et al., 2013). Fate mapping studies suggest that GATA4$^+$ neoplastic cells arise from a distinctive pool of WT1$^+$ progenitors in the adrenal capsule (Bandiera et al., 2013). Under basal conditions, these cells give rise to normal steroidogenic cells in the adrenal cortex; GDX activates these WT1$^+$ progenitors and triggers their differentiation into gonadal-like steroidogenic tissue. Thus, WT1$^+$ capsular cells represent a reserve stem/progenitor cell population that can be mobilized in response to extreme physiological demand (*i.e.*, GDX-induced hormonal changes). These WT1$^+$ capsular cells are presumed to be the progenitors of GDX-induced adrenocortical neoplasms. Whereas GATA4 drives GDX-induced adrenocortical neoplasia, GATA6 appears to inhibit the process. Conditional deletion of *Gata6* using *Sf1*-cre augments the GDX-induced expression of gonadal-like markers in mice (Pihlajoki et al., 2013).

GDX-induced adrenocortical neoplasia is a well documented phenomenon in not only mice but also hamsters, ferrets, goats, and other domesticated species (Beuschlein et al., 2012; Bielinska et al., 2009). Male Angora goats are routinely gonadectomized to enhance mohair production, and these castrate animals have an increased incidence of adrenocortical adenomas (Altman et al., 1969). GDX-induced adrenocortical neoplasia is a major cause of morbidity and mortality in pet ferrets, affecting up to 20% of these animals.

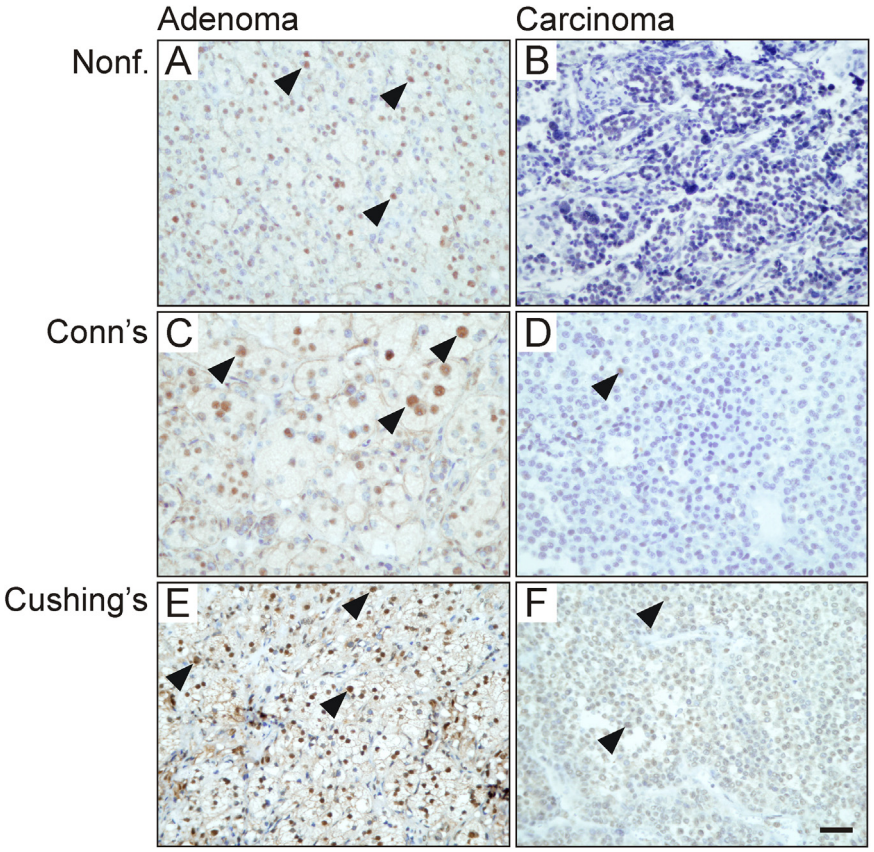

Fig. 5. Immunohistochemical staining of GATA6 in human adrenocortical tumors. Representative samples of nonfunctional (Nonf), Conn's (aldosterone-producing), and Cushing's (cortisol-producing) adenomas (A, C, E, respectively) and carcinomas (B, D, F, respectively) are shown. Immunoreactive cells (arrowheads) exhibit brown nuclear staining. Sections were counterstained with hematoxylin. Bar = 50 μm.

The neoplastic cells that accumulate in the adrenal glands of gonadectomized ferrets express GATA4 (Fig. 4) and other gonadal-like markers and secrete sex steroids (Bielinska et al., 2006; Schillebeeckx et al., 2015).

There are related mouse models in which GDX triggers the accumulation of gonadal-like cells in adrenal cortex. Following GDX, *Inha*-Tag mice develop malignant gonadal-like tumors, a process that is thought to entail a feed-forward signaling loop involving *Gata4* and *Lhcgr* (Rahman et al., 2004). Similarly, *Inha*$^{-/-}$ mice develop adrenocortical tumors in response to GDX (Matzuk et al., 1992). The resultant tumors exhibit increased expression of *Gata4* and other gonadal-like markers and a reciprocal down-regulation of *Gata6* (Looyenga and Hammer, 2006). Enforced expression of LH enhances adrenocortical neoplasia in *Inha*$^{-/-}$ mice (Beuschlein et al., 2003), whereas ablation of *Smad3* attenuates tumor growth in this model (Looyenga and Hammer, 2007).

4.3. Dysregulation of GATA factors in other genetically-engineered mouse models of adrenocortical neoplasia

Several mouse models of adrenocortical neoplasia exhibit abnormal expression of GATA factors, even in the absence of GDX. Mice harboring multiple copies of the steroidogenic factor-1 (*Sf1*) genetic locus, mimicking the amplification of *SF1* seen in childhood adrenocortical carcinoma, develop adrenocortical neoplasms that express gonadal-like markers including *Gata4* (Doghman et al., 2007). Activation of WNT/β-catenin signaling is a hallmark of human adrenocortical tumors (Assié et al., 2014; Tissier et al., 2005). Constitutive activation of β-catenin signaling, a hallmark of human adrenocortical tumors, triggers the accumulation of GATA4^{+} cells in the subcapsule of mice (Berthon et al., 2010). Similarly, overexpression of *Igf2*, a characteristic of human adrenocortical carcinomas, in the adrenal cortex of mice leads to the accumulation of subcapsular cells that express *Gata4* (Drelon et al., 2012). Collectively, these results suggest that deregulation of GATA factors is probably a general feature of adrenal tumorigenesis (at least in rodents), irrespective of whether it is triggered by GDX or genetic alterations also found in patients.

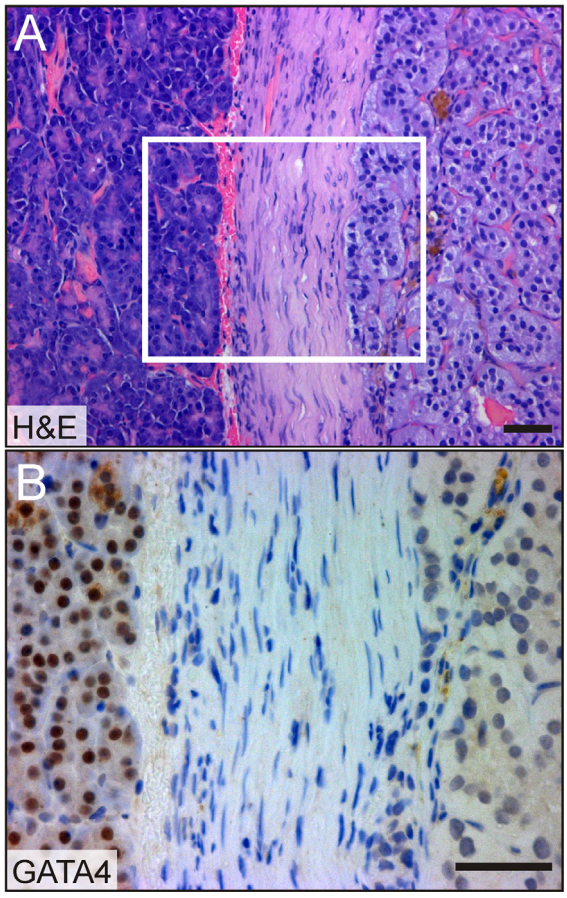

Fig. 7. GATA4 immunostaining distinguishes normal exocrine pancreas from a well-encapsulated insulinoma of the ferret. Serial sections of formalin-fixed tissue were stained with (A) hematoxylin and eosin (H&E), or (B) GATA4 antibody, followed by hematoxylin counterstaining. Note that pancreatic acinar cells exhibit GATA4 immunoreactivity, whereas insulinoma cells do not. Bar = 50 μm.

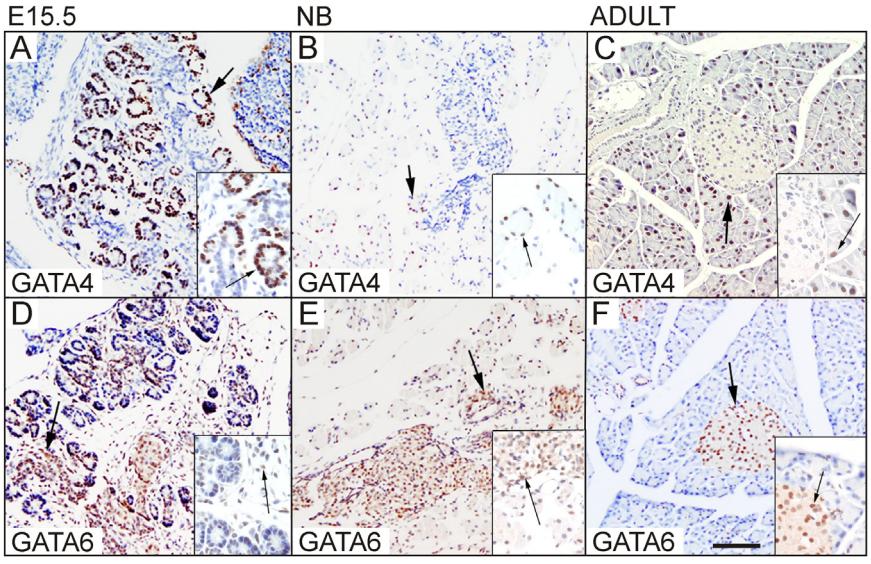

Fig. 6. Immunohistochemical staining of GATA4 and GATA6 in the developing mouse pancreas. Three developmental stages are illustrated: E15.5, newborn (postnatal day 1), and adult (3 months). The expression patterns reveal association of GATA4 (A, G, M) primarily with exocrine and GATA6 (B, H, N) with endocrine cells (arrows). Bar = 150 μm.

4.4. Expression of GATA4 and GATA6 in human adrenocortical tumors

The vast majority (>90%) of human adrenocortical adenomas and carcinomas express *GATA6* (Fig. 5) and approximately one-third express *GATA4* (Kiiveri et al., 2004). Diminished expression of *GATA6*, *SF1*, and their target gene *INHA* in adrenocortical carcinomas correlates with poor outcome (Parviainen et al., 2013). Overexpression of GATA6 in adrenocortical tumor cells enhances BMP signaling, which inhibits cell proliferation and viability (Johnsen et al., 2009). Although global DNA methylation surveys of human adrenocortical neoplasms have yielded inconsistent results, hypermethylation and downregulation of both *GATA6* and *GATA4* have been reported (Fonseca et al., 2012; Rechache et al., 2012).

5. Parathyroid neoplasms

5.1. Role of GATA3 in parathyroid development

The parathyroid glands regulate calcium balance in the body through the secretion of parathyroid hormone (PTH). *GATA3* expression has been reported in the second and third branchial arches, which harbor the progenitors of the parathyroid glands (Debacker et al., 1999; Grigorieva et al., 2010). The importance of GATA3 in parathyroid function has emerged from characterization of patients with hypoparathyroidism, sensorineural deafness, and renal anomaly (HDR) syndrome, a condition caused by heterozygous loss-of-function mutations in *GATA3* (Ali et al., 2007; Nesbit et al., 2004; Van Esch et al., 2000). Using mouse models of HDR syndrome, investigators have shown that GATA3 is involved in the embryonic development of the parathyroid glands and in adult parathyroid cell proliferation (Grigorieva et al., 2010). *Gata3*$^{-/-}$ and *Gata3*$^{+/-}$ embryos have smaller parathyroid–thymus primordia, and the parathyroid glands of adult *Gata3*$^{+/-}$ mice do not show increased proliferation or enlargement in response to hypocalcemia induced by a low calcium/vitamin D diet. These studies identified *Gcm2*, a gene required for proper parathyroid development, as a target of activation by GATA3.

5.2. GATA3 is a marker of parathyroid neoplasms

In two recent surveys of parathyroid tumors, GATA3 immunoreactivity was demonstrated in all parathyroid adenomas and carcinomas examined (Betts et al., 2014; Ordonez, 2014). As a tumor marker, GATA3 was found to be comparable in sensitivity and specificity to PTH, the marker that has traditionally been used in the diagnosis of parathyroid tumors.

6. Pancreatic neoplasms

6.1. Role of GATA4 and GATA6 in pancreatic development

The pancreas is a mixed endocrine and exocrine gland that arises from the amalgamation of dorsal and ventral buds of foregut endoderm. A number of transcription factor genes, including *Pdx1* and *Ptf1a*, have been shown to be essential for the early stages of pancreatic development in the mouse (Oliver-Krasinski and Stoffers, 2008). GATA4 and GATA6 exhibit overlapping patterns of expression in multipotential progenitor cells within the pancreatic anlagen (Decker et al., 2006; Ketola et al., 2004; Ritz-Laser et al., 2005). As development proceeds, GATA4 expression predominates in pancreatic acinar cells, while GATA6 predominates in the ductal compartment and a subpopulation of endocrine cells (Figs. 6 and 7) (Decker et al., 2006; Ketola et al., 2004).

GATA4 is able to transactivate the glucagon (*Gcg*) gene promoter *in vitro* (Ritz-Laser et al., 2005). The same study demonstrated that mutation of the GATA motif in the *Gcg* promoter reduces its basal promoter activity in glucagon producing cells. GATA6, but not GATA4, has been shown to physically interact with NKX2.2, an essential islet transcription factor (Decker et al., 2006).

The importance of GATA factors to pancreatic development was underscored when an international consortium of investigators demonstrated that heterozygous mutations in human *GATA6* cause a spectrum of pancreatic developmental defects ranging from agenesis to neonatal diabetes and adult-onset diabetes (Bonnefond et al., 2012; De Franco et al., 2013; Lango Allen et al., 2012; Yorifuji et al., 2012). Subsequently deletions or mutations of *GATA4* were shown to be a monogenic cause of neonatal and childhood-onset diabetes with variable exocrine phenotypes (Shaw-Smith et al., 2014). The roles of GATA4 and GATA6 in pancreatic development and disease have been modeled in the mouse (Carrasco et al., 2012; Decker et al., 2006; Martinelli et al., 2013; Watt et al., 2007; Xuan et al., 2012). Expression of a GATA6-Engrailed dominant repressor fusion protein in pancreatic progenitors using a *Pdx1* promoter caused pancreatic hypoplasia (Decker et al., 2006). Analysis of embryos derived by tetraploid complementation of *Gata4*$^{-/-}$ ES cells demonstrated a complete absence of the ventral but not the dorsal pancreas; *Gata6*$^{-/-}$ embryos displayed a similar, albeit less dramatic, phenotype (Watt et al., 2007). Conditional mutagenesis of either *Gata4* or *Gata6* in multipotent pancreatic progenitors (using *Pdx1*-cre) has minimal impact on pancreatic development or function, whereas mutagenesis of both genes results in pancreatic agenesis and diabetes (Carrasco et al., 2012; Xuan et al., 2012). The double mutant mice exhibit impaired proliferation of pancreatic progenitor cells, aberrant branching morphogenesis, and a subsequent failure to induce the differentiation of progenitor cells expressing *Cpa1* and *Neurog3*. The prevailing hypothesis, based on the established roles of GATA4 and GATA6 in other foregut derivatives such as the lung and intestine (Beuling et al., 2012; Zhang et al., 2008), is that GATA proteins, in combination with other transcription factors, regulate the balance between stem cell expansion and differentiation in the developing pancreas. Although GATA6 is preferentially expressed in endocrine pancreas, conditional knockout mouse studies using *Ptf1a*-cre have shown that GATA6 has direct effects on survival of acinar cells in the exocrine pancreas (Martinelli et al., 2013). More recently, investigators have generated β-cell specific knockouts of *Gata4* or *Gata6* and concluded that these factors have important but nonessential roles in promoting endoplasmic reticulum integrity and β-cell survival, which may contribute to the pathogenesis of type 1 diabetes (Sartori et al., 2014). The precise function of GATA4 in mature pancreatic acinar cells is unclear.

6.2. Gata6 is downregulated in insulinomas triggered by MEN1 deficiency in the mouse

Mice harboring loss-of-function mutations in *Men1* develop multiple endocrine neoplasias (see Section 3.4) including insulinomas (Serewko-Auret et al., 2010). Transcriptome analysis of isolated control, normal, hyperplastic, and adenomous islets showed that *Gata6* downregulation accompanies tumor formation. It has been proposed that *Gata6* dysregulation plays a fundamental role in tumor formation and progression in this model by modulating TGFβ superfamily or WNT/β-catenin signaling, as in other systems (see Section 1.3).

6.3. Roles of GATA6 and GATA4 in pancreatic ductal adenocarcinoma

Although not an endocrine tumor, pancreatic ductal

Table 5
GATA factors in endocrine cell types and their corresponding neoplasms.

Tissue	GATA factor	Cell type	Tumor
Ovary	GATA4	Granulosa cells	**Human:**
		Theca cells	AGCT, SLCT, thecoma–fibroma
	GATA6	Granulosa cells	
		Theca cells	
		Corpus luteum	
Testis	GATA1	Sertoli cells	
	GATA4	Sertoli cells	**Human:**
		Leydig cells	LCCSCT
		Peritubular cells	**Canine:**
			SCT, LCT, MGSCT
	GATA6	Sertoli cells	
		Leydig cells	
Adrenal cortex	GATA4	Fetal cortex	**Mouse, ferret**:
			GDX-induced neoplasms
			Mouse:
			Inhα-Tag and *Inha*$^{-/-}$ gonadal-like tumors
			Human:
			Minority of adenomas and carcinomas
	GATA6	**Mouse**:	**Human:**
		Fetal cortex	Majority of adenomas, carcinomas
		Adult (sub)capsule	
		Human:	
		Fetal cortex	
		zR	
Parathyroid gland	GATA3	Parathyroid cells	**Human:**
			Adenomas and carcinomas
Pancreas	GATA4	Multipotential progenitor cells	**Human:**
		Acinar cells	Pancreatic ductul adenocarcinoma
	GATA6	Multipotential progenitor cells	**Human:**
		Islet cells	Pancreatic ductul adenocarcinoma
Pituitary	GATA2	Gonadotrope cells	**Human:**
		Thyrotrope cells	Adenomas

Abbreviations: AGCT, adult-type granulosa cell tumor; GDX, gonadectomy; LCCSCT, large-cell calcifying Sertoli tumor; LCT, Leydig (interstitial) cell tumor; MGSCT, mixed germ cell sex cord-stromal tumor; SCT; Sertoli cell tumor; SLCT, Sertoli–Leydig cell tumor; zR, zona reticularis.

adenocarcinoma (PDA) sheds light on the role of GATA factors in organogenesis and oncogenesis. PDA has a complex genomic landscape characterized by frequent point mutations and copy number changes. Common genetic changes include activating mutations of *KRAS2* and inactivating mutations in the cell cycle regulator *CDKN2A*, the tumor suppressor *TP53*, and *SMAD4* (Hong et al., 2011; Jones et al., 2008). *GATA6* amplification and overexpression are hallmarks of PDA (Collisson et al., 2011; Fu et al., 2008; Kwei et al., 2008). Enforced expression of GATA6 in pancreatic cancer cell lines enhances proliferation and growth in soft agar, whereas inhibition of GATA6 impairs growth of pancreatic cancer cell lines. GATA6 activates signaling in pancreatic cancer by negatively regulating the WNT antagonist Dickkopf-1 (DKK1) (Zhong et al., 2011). Smoking is a major risk factor for pancreatic cancer, and a recent study showed that nicotine promotes progression of *Kras*-induced pancreatic adenocarcinoma via *Gata6*-dependent dedifferentiation of acinar cells (Hermann et al., 2014). *GATA4* is frequently overexpressed and infrequently methylated in PDA, whereas *GATA5* is generally hypermethylated in these neoplasms (Fu et al., 2007). A separate study documented GATA4 immunoreactivity in a majority of infiltrating pancreatic adenocarcinomas (Karafin et al., 2009).

7. Pituitary neoplasms

7.1. Role of GATA2 in pituitary development and function

Among differentiated hormone-secreting cell types found in the pituitary gland, both gonadotrope and thyrotrope cells express *Gata2* from E10.5 onward in the mouse. The secretory products of thyrotrope and gonadotrope cells are heterodimers that share a common α-glycoprotein subunit (αGSU) and a specific β-subunit (FSHβ, LHβ, and thyrotropin-β) (reviewed in Viger et al., 2008). The genes encoding αGSU (*Cga*) and thyrotropin-β (*Tshb*) are targets of activation by GATA2 (Gordon et al., 1997; Steger et al., 1994). This transcription factor has also been implicated in the regulation of the GnRH receptor gene (Schang et al., 2013).

Analyses of transgenic and knockout mice have established that GATA2 is involved in both gonadotrope and thyrotrope development (Charles et al., 2006; Dasen et al., 1999; Scully and Rosenfeld, 2002). Interactions between GATA2 and another transcription factor, PIT1, are critical determinants of pituitary cell fate (Dasen et al., 1999; Scully and Rosenfeld, 2002). In gonadotropes, where GATA2 is expressed in the absence of PIT1, GATA2 promotes the expression of gonadotrope-specific genes. In thyrotropes, where GATA2 and PIT1 are coexpressed, thyrotrope-specific genes are up-regulated by the binding of both factors to adjacent DNA *cis*-elements. PIT1 interacts via its homeodomain with a zinc finger of GATA2, modulating target gene transactivation (Dasen et al., 1999). Conditional ablation of *Gata2* in the anterior pituitary reduces gonadotrope and thyrotrope cell numbers at birth and impairs the secretory capacity of these cells in the adult (Charles et al., 2006).

7.2. GATA2 is a marker of pituitary neoplasms

Consistent with its established role in gonadotrope and thyrotrope development GATA2 is found in most αGSU-positive and thyrotropin-secreting human pituitary adenomas (Umeoka et al., 2002; Wang et al., 2009).

8. Summary and outlook

Studies over the past two decades have established that GATA factors are required for the proper development, differentiation, and function of endocrine tissues. More recently, GATA factors have been implicated in forms of endocrine neoplasia (Table 5), although the molecular mechanisms involved are not fully understood. Altered GATA factor expression or function owing to acquired genetic (mutations, deletions, amplifications) or epigenetic changes (*e.g.*, DNA methylation) has been linked to tumor formation. GATA factors can impact tumorigenesis through modulation of key developmental signaling pathways implicated in oncogenesis, such as the WNT/β-catenin and TGFβ signaling pathways. In addition to affecting signaling pathways, GATA factor dysregulation can have effects on tumor cell metabolism, ploidy, and invasiveness.

Traditionally, transcription factors have been considered poorly druggable, but recent studies offer hope that GATA factors can be targeted pharmacologically in endocrine neoplasms. A small molecule inhibitor of GATA2 has been shown to suppress AR expression and exert anticancer activity against prostate cancer cell lines (He et al., 2014). It may be feasible to adopt similar approaches for inhibition of GATA factors in other tumors.

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Testicular Steroidogenic Cells to the Rescue

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In this issue of *Endocrinology*, Padua et al (1) characterize the phenotype of mice lacking both GATA4 and GATA6 in steroidogenic cells. The double-mutant mice are born with adrenal aplasia. The female pups die from adrenocortical insufficiency, but the males survive owing to ectopic corticoid production by adrenal-like cells in the testis. These mutant mice shed new light on the regulation of steroidogenic cell differentiation and may provide a model for the study of testicular adrenal rest tumors (TARTs).

Steroidogenic cells in the adrenal cortex and testis arise from a common pool of progenitors in the adrenogonadal primordium (AGP), a specialized group of coelomic epithelial cells in the urogenital ridge (Figure 1) (2). Adrenocortical progenitors migrate medially and combine with sympathoblasts, the precursors of the medulla, to form the nascent adrenal gland, which begins to produce glucocorticoids and other steroids (3). Gonadal progenitors migrate laterally and combine with primordial germ cells to form the bipotential gonad. Expression of *Sry* in the male gonad triggers the differentiation of Sertoli cells, which nurture germ cells and secrete paracrine factors that promote the differentiation of steroidogenic Leydig cells (4). In addition to producing T that is crucial for masculinization of the male fetus, fetal Leydig cells secrete insulin like-3, a hormone that promotes testicular descent (5).

The common developmental origin of the adrenal cortex and testis is reflected in overlapping functional profiles for these organs. For example, the testes of newborn mice contain interstitial cells that express adrenocortical differentiation markers (eg, *Cyp21a1*, *Cyp11b1*), and ACTH stimulates androgen and glucocorticoid production by this tissue (6–8). Conversely, the adrenal gland of the adult mouse harbors rare stem/progenitor cells that can differentiate into gonadal-like cells in response to the hormonal changes that accompany gonadectomy (9–11).

Among the plethora of transcription factors implicated in the differentiation of adrenal or testicular steroidogenic cells, a few are indispensable (3, 12). The prototype of these is steroidogenic factor-1 (SF1; also called Ad4BP or NR5A1). *Sf1* is expressed in the AGP, adrenocortical cells, Sertoli cells, and Leydig cells. Mice lacking SF1 exhibit defects in both adrenal and testicular development (13, 14). Because SF1 is expressed in all steroidogenic tissues, it cannot by itself account for functional differences between steroid-producing cells in the adrenal cortex and testis. Two members of the GATA transcription factor family, GATA4 and GATA6, also regulate steroidogenesis and have been shown to impact the balance between adrenal and gonadal differentiation (11). During fetal mouse development, *Gata4* and *Gata6* are coexpressed in adrenocortical cells, Sertoli cells, and Leydig cells (15, 16). After birth, *Gata6* expression persists in the adrenal cortex and testis, but *Gata4* expression wanes in the adrenal gland (15, 16).

Mice harboring germline homozygous null mutations in either *Gata4* or *Gata6* die early in embryonic development, so Cre-LoxP technology has been used to investigate the roles of GATA4 and GATA6 in steroidogenic tissues (15). These studies have shown that GATA4 is required for genital ridge development, testicular morphogenesis, and fetal/adult Sertoli cell function (17–19). In contrast, loss of GATA6 in steroidogenic cells mainly impacts adrenocortical zonation and function (20). As detailed in this issue of *Endocrinology*, combined loss of GATA4 and GATA6

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Abbreviations: AGP, adrenogonadal primordium; cKO, conditional knockout; SF1, steroidogenic factor-1; TART, testicular adrenal rest tumor.

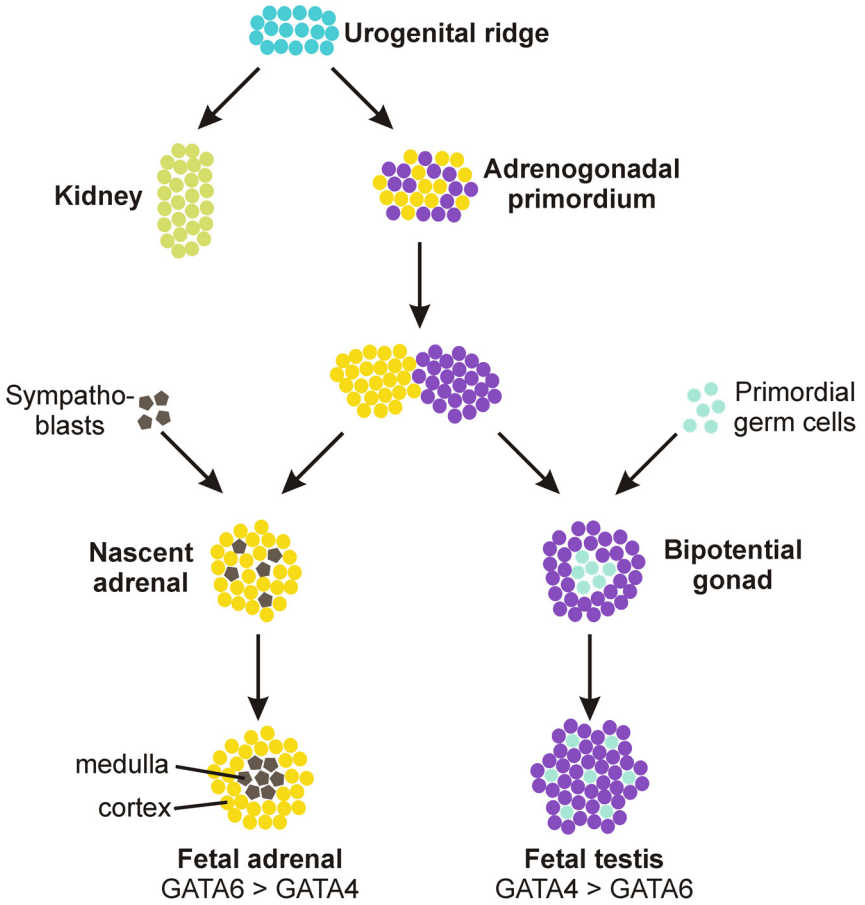

Figure 1. Development of adrenal gland and testis. The adrenal cortex and testis arise from a common progenitor, the adrenogonadal primordium, a specialized region of the urogenital ridge. Two members of the GATA transcription factor family, GATA4 and GATA6, are expressed in the fetal adrenal and testis.

in $SF1^+$ cells causes striking defects in both adrenal and testicular development (Figure 2) (1). The double-mutant mice lack adrenal glands. The female mutants die due to adrenocortical insufficiency. The males survive and exhibit a dramatic gonadal phenotype that includes severe testicular hypoplasia, cryptorchidism, disorganized seminiferous tubules, germ cell depletion, Leydig cell loss, and impaired T production. Intriguingly, nests of cells that express adrenocortical markers (*Cyp21a1*, *Cyp11b1*, *Cyp11b2*, and the ACTH receptor *Mc2r*) accumulate in the testes of double-mutant mice postnatally and persist into adulthood, suggesting that males escape death because of ectopic adrenocorticoid production in the testis.

The adrenocortical-like cells that accumulate in the testes of the double-mutant males resemble TARTs, neoplastic nodules of hormonally active adrenocortical tissue that develop in men with CYP21 or CYP11B1 deficiency (21–23). These neoplasms are benign but can obstruct the rete testis, leading to testicular degeneration and infertility. TARTs are thought to arise from multipotential stem/progenitor cells that reside in the testis (6). RNA analysis has shown that TARTs express adrenocortical differentiation markers (*CYP11B1*, *CYP11B2*, *MC2R*) at 1000–10 000 higher levels than adjacent testicular tissue (23). TARTs also express Leydig cell markers, such as the LH/chorionic gonadotropin receptor (23). The endocrine and paracrine factors that drive TART formation are not fully understood. Elevated ACTH levels can promote TART growth, but a longitudinal analysis of men with 21-hydroxylase deficiency found no association between the presence of TARTs and parameters of disease control with exogenous glucocorticoids ± mineralocorticoids (24). Thus, other signaling molecules such as angiotensin II and gonadotropins are presumed to be involved in tumorigenesis. The presence of LH/chorionic gonadotropin receptor in TARTs may explain why growth of these tumors often coincides with the onset of puberty. *Gata4*/*Gata6* double-mutant mice afford an experimentally tractable model to investigate the signals underlying TART formation and growth.

The phenotype of the double mutants raises a paradox. If the combination of GATA4 and GATA6 is essential for steroidogenic cell differentiation in the adrenal cortex, how can adrenocortical-like cells arise in the testes of the double mutants? Have the heterotopic cells evaded complete Cre-mediated recombination of *Gata4* and *Gata6*? Does the testis have an alternative route of adrenocortical cell differentiation that circumvents the need for *Gata4* and *Gata6*? Future experiments are likely to address this conundrum and explore the mechanistic basis for the phenotypic abnormalities in the double-mutant mice, including identification of genetic targets for GATA factors in steroidogenic cells. Studies in different cell types have implicated GATA4 and GATA6 in not only steroid production but also a vast array of other cellular processes, such as energy metabolism, apoptosis, polarization, and cytokinesis (25–33). By recruiting functionally distinct classes of transcriptional cofactors to promoter/enhancer sites in DNA, GATA factors can either activate or repress gene expression, depending on the context (34).

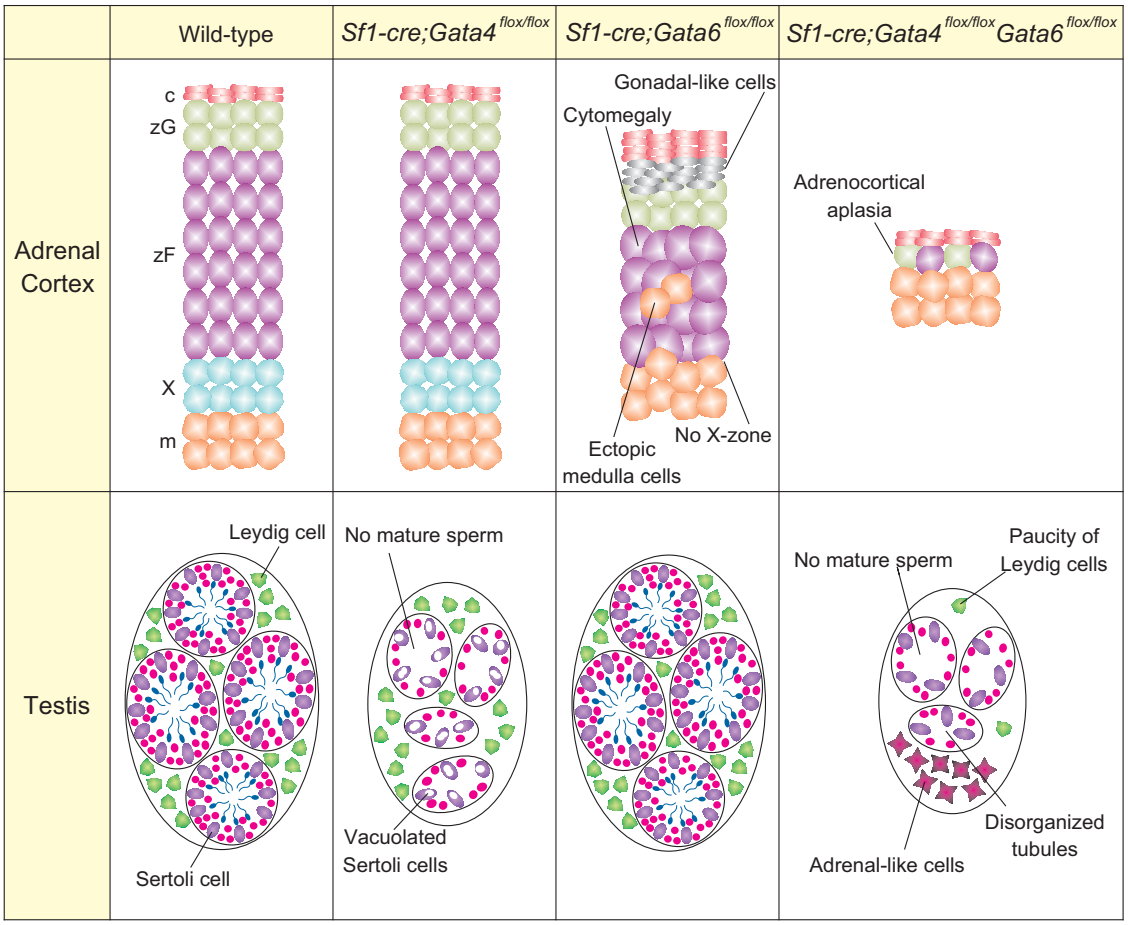

Figure 2. Phenotypes of single and double conditional knockout (cKO) mice generated with *Sf1*-cre. Adult *Gata4* cKO mice are undervirilized (hypoplastic penis, decreased anogenital distance) and have small testes that lack mature sperm (18). Sertoli cells in these mice are vacuolated and exhibit functional defects including reduced expression of *Dmrt1*, a sex determination gene. The adrenal glands of adult *Gata6* cKO mice have a thin, cytomegalic cortex and accumulate gonadal-like cells in the subcapsule (20). Other structural abnormalities include ectopic medulla cells and absence of the X-zone, a remnant of the fetal cortex. *Gata4*/*Gata6* double mutants are born with adrenocortical aplasia (1). Female mice die due to adrenocortical insufficiency. Male mice survive but are infertile and have small, partially descended testes with disorganized seminiferous tubules, deranged spermatogenesis, and decreased numbers of Leydig cells. T production is reduced. Nests of adrenal-like cells accumulate in the testes of these mice and are thought to be the source of life-saving corticoids. c, capsule, m, medulla; X, x-zone; zF, zona fasciculata; zG, zona glomerulosa.

In summary, the findings of Padua et al (1), coupled with prior reports (20, 30, 35, 36), show that GATA4 and GATA6 function synergistically during steroidogenic cell development and play key roles in establishing the cellular identities of adrenocortical and gonadal cells. The *Gata4*/*Gata6* double mutant expands the list of mouse models exhibiting ectopic development of steroid-producing cells (11); such models have proven invaluable in elucidating the molecular regulation of steroidogenic cell fate. From a therapeutic perspective, testicular progenitors with the capacity for adrenal-like differentiation represent a population of developmentally primed cells that could be manipulated to reconstitute function in patients with adrenocortical failure.

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